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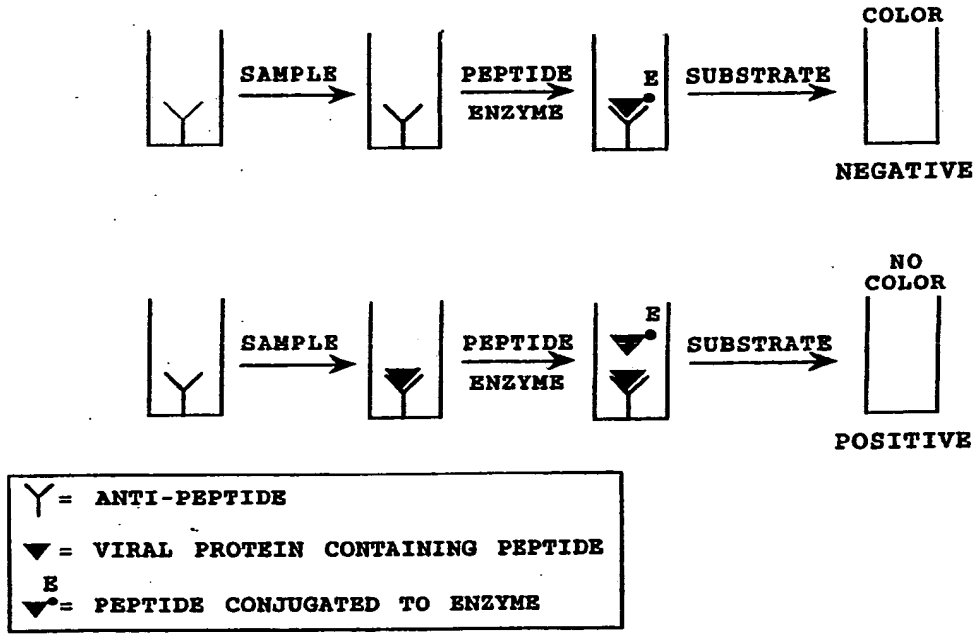


(57) Abstract

Peptide antigens which are immunoreactive with sera from individuals infected with hepatitis C virus (HCV) are disclosed. Several of the antigens are immunologically reactive with antibodies present in individuals identified as having chronic and acute HCV infection. The antigens are useful in generating antibodies for use in antigen-inhibition immunodiagnostic methods for detecting the presence of HCV antigens in test samples. The antigens are useful in diagnostic methods for detecting HCV infection in humans and for vaccine development.

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# PEPTIDE BASED HEPATITIS C VIRUS IMMUNOASSAYS

## Field of the Invention

The present invention relates to synthetic peptides which contain amino acid sequences of polypeptides encoded by the RNA genome of the etiologic agent of Non-A Non-B Hepatitis (NANBH), herein referred to as Hepatitis C Virus (HCV). The synthetic peptides of the present invention have applications in diagnostics and vaccine development.

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**Background of the Invention**

Non-A Non-B hepatitis is a generic term used for cases of viral hepatitis in which Hepatitis A Virus (HAV) and Hepatitis B virus (HBV) have been excluded. Other viruses that may play a role in viral hepatitis disease are Hepatitis Delta Virus (HDV), Cytomegalovirus (CMV), and Epstein Barr virus (EBV) (Hollinger, 1990). NANBH accounts for 80-90% of the cases of transfusion-associated hepatitis recorded annually in the United States. In addition, as much as 20% of sporadic outbreaks of hepatitis may be caused by the Non-A Non-B viral agent(s) which are implicated in such disease outbreaks.

Clinical and epidemiologic parameters of NANBH are similar to those noted for Hepatitis B except that chronic infections appear to occur more frequently with NANBH cases: it has been estimated that 10-70% (mean of 54%) of people diagnosed with NANBH develop a chronic infection (Dienstag, 1983). Similar levels of chronicity have been noted in experimentally infected chimpanzees (Burk et al. 1984a).

The blood derived from many of these chronically infected individuals and chimpanzees has been proven to be infectious even though the host remains asymptomatic (Burk et al., 1984a; reviewed in Hollinger, 1990). Tubules, cylindrical structures, undulating membranes and protein complexes are characteristically observed by electron microscopic observation of liver tissue derived from infected humans and chimpanzees (Burk et al., 1984; reviewed in Hollinger 1990). In the absence of specific serologic markers such unique cytoplasmic structures observed with the aid of

electron microscopy have been accepted as a hallmark for NANBH. However, these changes may only represent a cellular reaction to the infectious process and thus may have little or no relationship to viral components. Diagnosis of NANBH until recently was based on biochemical evidence after serologic exclusion of HAV and HBV.

Before recent developments concerning the identification of an etiologic agent for NANBH the most meaningful predictor of potential infectivity for a given blood donor was the ALT value (Hollinger, 1984). Transfusion of a donor unit with an ALT value exceeding 45 IV/liter is significantly associated with the risk of contracting NANBH. A second surrogate test is the anti-HBc assay (Alter, 1988) based on the assumption that this will identify individuals who are in high risk populations with a greater opportunity to be infected with either infectious agent, i.e., with HBV or Non-A Non-B. The identification of a Non-A Non-B Hepatitis infectious agent, now referred to as Hepatitis C Virus (HCV), was made by two independent research teams, whose scientific finds were recently published (Choo et al., 1989a; Jacob et al., 1990a).

Lanford et al., U.S. Patent Application Number 07/222,569, filed 21 July 1988, herein incorporated by reference, developed a defined serum-free media that sustains long-term *in vitro* cultures of differentiated primate hepatocytes. Using hepatocytes, obtained from an HCV acutely infected chimpanzee, cultured in this *in vitro* tissue culture system Jacob et al. (1990a) demonstrated replication of HCV in primary chimpanzee hepatocytes. The source of the infectious NANBH inoculum was the Hutchinson strain (Feinstone et al. 1981). The

presence of HCV in test cell supernatant fluids was supported as follows: 1) by infection of a normal chimpanzee using the test cell supernatant fluids; 2) by observation of enveloped 39-60nm virion particles; and 3) by sequencing of cloned viral RNA. This work is being extended (Jacob et al. (1990B)) to demonstrate HCV replication in chimpanzee hepatocytes inoculated with an HCV stock derived from tissue culture media.

Concurrent with the above investigations Chou et al. (1989) constructed recombinant cDNA libraries from chimpanzee plasma from animals infected with an infectious NANBH Factor VIII concentrate. A clone, referred to as 5-1-1, which reacted with a serum derived from a NANBH patient was identified in these libraries. Subsequent studies showed that clone 5-1-1 was derived from a positive single-stranded RNA molecule containing roughly 10,000 nucleotides (Chou et al., 1989). A recombinant protein, which included 5-1-1 coding sequences, was synthesized for expression in yeast. This fusion protein was constructed from coding sequences derived from 3 overlapping HCV clones and coding sequences for human super oxide dismutase (SOD). The resulting fusion protein was designated C-100 (Houghton et al. 1989). C-100 contains amino acids 1569-1931 relative to the HCV polyprotein sequence reported in Houghton et al. (1990). A HCV solid phase radioimmunoassay (RIA) was developed with the recombinant protein and was used to test a panel of human sera (Kuo et al. 1989). Using this RIA, the peptide designated C-100 was found to react immuno-specifically with up to 80% of chronic NANBH samples, and about 15% of acute NANBH samples (Kuo et al.).

Based on the morphology of infectious tissue culture derived virions (Jacob et al., 1990a, 1990b) and the nucleotide sequence of the assumed non-structural coding region of the single stranded RNA genome (Chou et al. 1989), it appears that HCV is a relative of the Flavivirdae or Togaviridae viruses. These characteristics are consistent with earlier studies that (i) predicted HCV particle size by selective ultrafiltration (He et al., 1987) and (ii) demonstrated that HCV infectivity was sensitive to chloroform treatment (Bradley et al. 1983; Feinstone et al. 1983).

A number of reports have compared sequence homologies of cDNA fragments of HCV derived from human plasma (Kubo et al. 1990), from the Hutchinson strain propagated in primary chimpanzee hepatocytes (Jacob et al. 1990B), and from the original Hutchinson inoculum (Miller et al., 1990). Kubo et al. (1990) reports that a cDNA fragment isolated from a Japanese blood donor showed 79.8% homology at the nucleotide level and 92.2% homology at the amino acid level to the prototype HCV cDNA as originally described by Choo et al. (1989). Jacob et al. reports that the nucleotide sequence derived from Hutchinson strain HCV has a nucleotide homology of 79.8% and an amino acid homology of 93.8% as compared to the J1 sequence (determined by Kubo et al. 1990), but a higher level of nucleotide sequence homology, 96.2%, observed with the PT strain (described by Kubo et al. 1990). These reports indicate that individual isolates of HCV may have marked sequence divergence.

Kuo et al. (1989) reported that studies using the C-100 based immunoassay for detection of antibody in post-transfusion hepatitis patients

indicated that HCV is the major cause of transfusion-associated NANBH throughout the world as well as community-acquired NANBH in which no parenteral exposure to the virus has been made.

A number of studies have recently appeared confirming the efficacy of using the C-100 RIA test for screening sera suspected of HCV infection (Alter et al., 1990; Miyamura et al., 1990; Mosley et al., 1990). These studies suggest that HCV is the etiologic agent for 50-90% of transfusion associated non-A non-B hepatitis: particularly those cases which develop chronicity. The antibody activity detected by the current anti-HCV assay (Kuo et al. 1989) is uniformly detected after acute infection following the peak elevation of ALT values (Alter et al. 1990; Figure 4). To verify exposure to HCV using the C-100 based detection system, a later blood sample should be tested since seroconversion may not be noted for periods of up to a year (Alter et al. 1990).

Infection during the acute-phase window during which seroconversion, as detected by use of the C-100 antigen, has not taken place may be detected using a recently developed HCV RNA-specific polymerase chain reaction (Weiner et al. 1990; Carson et al. 1990). Using this polymerase chain reaction HCV RNA has been detected in acute phase liver tissue and blood samples which are either (i) antibody negative acute phase (based on reaction with C-100) or (ii) blood obtained during the chronic phase where antibody to C-100 is undetectable.

An epidemiologic survey by Stevens et al. (1990) indicates that anti-C-100 antibodies are present in 0.9 to 1.4% donors. The incidence rates

were higher in Black and Hispanic donors than in white donors. The prevalence of anti-C-100 antibodies correlated with increasing age through the age of 40, but decreased thereafter. This observation indicates loss of detectable antibody with time.

The antibody detected by the present C-100-based ELISA is directed to an epitope presumably encoded by the non-structural regions of the HCV genome (Choo et al. 1989). Alter et al. (1990) established that serum positive for the anti-C-100 antibodies was infectious. Accordingly, antibodies directed against the epitope defined by C-100 would not appear to provide protection against re-infection by HCV. Analysis of sequential serum samples from prospectively-followed transfusion recipients indicates that the development of antibody to C100-3 is considerably delayed (Alter *et al., 1990*). In many infected individuals there is a 8 to 20 week period between ALT rises and development of antibody.

First generation HCV assays used NS4 (termed C100-3) antigen fused to superoxide dimutase (SOD) as the capture solid phase antigen (Kuo *et al., 1989*). Subsequently, a second generation along with confirmatory tests have been developed which include NS3 (C33c) and capsid (C22) proteins in addition to the C100-3 antigen (Van der Poel *et al.*, 1991).

**Summary of the Invention**

The present invention relates to a method for detecting the presence of viral antigens in a sample. In the method a sample is contacted with at least one antibody which is reactive with a viral HCV antigen. Typically, the antibody is attached to

a solid support such as the inside surface of the wells of a multiwell plastic plate or a protein binding membrane, like nitrocellulose. The antibody is then examined for the presence of bound viral antigen. This can be accomplished by reacting the solid support with an antigen-reporter complex, where the viral antigen competes with the binding of the antigen-reporter complex to the antibody. The level of antigen-reporter complex which is bound to the solid support is then evaluated. The method of the present invention is described in detail for application to the detection of HCV antigens in a sample.

The method of the present invention permits the quantitation of viral antigen level based on the level of viral antigen inhibition of the binding of antigen-reporter complex to the antibody.

A number of sample types can be evaluated by the method of the present invention including tissue culture medium and serum samples (e.g., chimpanzee serum and human serum).

In one embodiment of the present invention, a method for detecting the presence of hepatitis C virus (HCV) antigens in a sample, polypeptide antigens used to generate the antibodies useful in the method of the present invention may contain the following antigenic sequences: SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:24, SEQ ID NO:14 and SEQ ID NO:16.

The polypeptide antigen of the antigen-reporter complex typically contains the antigenic sequence used to generate the antibody used in the method.

Two or more antibodies can be used in the method of the present invention. In a preferred embodiment where two antibodies are used,

corresponding polypeptide antigens are used, for example, the antigen of the first antigen-reporter complex may contain SEQ ID NO:7 and the antigen of the second antigen-reporter complex may contain SEQ ID NO:16.

A number of reporter moieties can be used in the antigen-reporter complex including, enzymatic reporters, radioactive reporters, fluorescent reporters. In one embodiment of the invention the enzymatic reporter horse radish peroxidase coupled with a colorametric substrate such as 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS).

In one embodiment of the present invention, the method is used for detecting the presence of hepatitis C virus (HCV) antigens in the presence of HCV antibodies in a sample obtained from an HCV-infected host. In this method, at least one antibody is generated that is capable of binding an HCV antigen that is not normally immunoreactive with sera from the HCV-infected host. The serum sample from the infected host is contacted, in a reaction mixture, with the antibody under conditions that promote the binding of the antibody and the HCV antigen that is not normally immunoreactive with sera from the HCV-infected host. Typically, the antibody is attached to a solid support and a non-ionic surfactant, such as a polyoxyethylene sorbitan, is included in the reaction mixture. The antibody is then examined, as described above, for the presence of bound HCV antigen by reacting the solid support with an antigen-reporter complex, where the antigen and the antigen-reporter complex compete for binding to the antibody. The presence of the antigen-reporter complex on the solid support is then detected as described above. One exemplary

antigen for use in this method is the polypeptide presented as SEQ ID NO:16.

The invention also includes a diagnostic kit for use in screening samples for the presence of hepatitis C virus (HCV) antigens. The kit contains at least one antibody which is reactive with an HCV antigen and an antigen-reporter complex, where the HCV antigen competes with binding of the antigen-reporter complex to the antibody. In the kit the antibody can also be attached to a solid support. Examples of antigens of the antigen-reporter complex that are useful in the present invention are polypeptides containing the following antigenic sequences: SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:24, SEQ ID NO:14 and SEQ ID NO:16. The reporter moieties can be selected to have a number of properties, as described above.

In another aspect, the invention includes an HCV polypeptide antigen characterized by:

(a) an epitope formed by the sequence presented as SEQ ID NO:3; and

(b) effective to recognize, by immunoreactivity with HCV-specific antibodies in human HCV anti-sera, a substantially broader range of HCV anti-sera than is recognized by a 362 amino acid HCV peptide having a 23 amino acid N-terminal region amino acid overlap with the SEQ ID NO:3.

The region of overlap of the peptide antigen with the 362 amino acid HCV peptide may be substantially no greater than 23 amino acids; that is, the peptide antigen may terminate at its N-terminus at the first of 23 overlapping amino acids, or may contain non-HCV sequences upstream (in the N-terminal direction) at the first of the 23 overlapping amino acid sequences. The epitope may

be formed by the peptide defined by the actual presented sequence.

In another aspect, the invention includes a polypeptide antigen consisting essentially of amino acid residues contained in one from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.

Also forming part of the invention is a diagnostic kit for use in screening human blood containing antibodies specific against hepatitis C virus (HCV) infection. The kit includes an HCV polypeptide antigen of the type identified above, and a system for detecting the binding of HCV-specific serum antibodies to the antigen.

In one embodiment of the kit, the detecting system includes a solid support to which the antigen is attached and a reporter-labeled anti-human antibody, where binding of HCV-specific serum antibodies to the antigen is detected by binding of the reporter-labeled antibody to the solid surface.

The kit is used in practicing a diagnostic method which forms another part of the invention. In this method, serum from an individual suspected of infection by HCV is reacted with an HCV polypeptide antigen of the type identified above, forming an antigen-antibody complex. The reaction products are then examined for the presence of such complex.

In still another aspect, the invention includes purified antibodies for use in the method and kits of the present invention, in particular, purified antibodies immunoreactive with a polypeptide containing the following antigenic sequences: SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:24, SEQ ID NO:14 and SEQ ID NO:16. The

antibodies of the present invention may be polyclonal or monoclonal.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

**Brief Description of the Figures**

Figure 1 illustrates the clinical course of NANBH followed after infection with HCV. There is an initial acute phase during which virus can be detected by passage into non-immune animals, liver damage diagnosed by elevation of a liver specific enzymes, alanine aminotransferase, in the blood stream and by symptomology in many patients. During stage 1, a method such as direct detection of virus antigen is required to diagnose infectivity. After this acute phase of HCV infection 60-70% of HCV-infected patients develop a chronic phase where they remain infected with HCV, sometimes for life. During this period, two assays are useful, (i) a test to detect non-protective antibody and (ii) a test that will detect circulating immune complexes containing the HCV virus, or viral subunits complexed to antibody. After chronic infection some patients resolve the chronic infection and produce protective antibody. An assay to detect protective "convalescence" antibody would be of value in the clinical management of these patients.

Figure 2 shows an acid sucrose gradient profile of an acidified immune complex prepared by mixing acute phase NANBH chimpanzee plasma with a convalescent phase NANBH-infected chimpanzee plasma.

Figure 3 presents an autoradiogram of a gel on which was separated radiolabeled, immune complexed purified material, derived from acute phase plasma, from chimpanzee 450 vs. chimpanzee 159 (lane 1), and plasma from acute phase chimpanzee 450 vs. chimpanzee 92 (lanes 2 and 4). Pre-bleed plasma derived from chimpanzee 92 vs. chimpanzee 159 was also complexed and purified by the same procedure (lanes 3 and 5). The antibody positive plasma from chimpanzee 159 was convalescent plasma.

Figure 4 presents an autoradiogram of iodinated preparations purified by immune complexing. Plasmas derived from pre-bleed and acute phase chimpanzee No. 341 were complexed with convalescent chimpanzee No. 92. Pre-bleed sample (Lane 1), acute phase plasma, day 67 (lane 2) day 135 (lane 3) and day 139 are shown.

Figure 5 presents an autoradiogram of iodinated immune complex purified preparations. Materials analyzed on this gels were derived from chimpanzee 325 plasma obtained from pre-bleed (lane 1), and the acute phase, day 57 (lane 2), day 106 (lane 3), day 112 (lane 4), day 57 (lane 5), pre-bleed (lane 6), and from chimpanzee 92 pre-bleed (lane 7), and day 21 (lane 8). Each was complexed to convalescent antibody positive plasma from chimpanzee 92.

Figure 6 presents an autoradiogram of pre-bleed (Pb) and acute (Ac) chimpanzee 450 plasma purified by salt precipitation and high salt elution from DEAE-52 Sepharose column. The eluted material was iodinated using enzymobeads (lanes 1 and 5; Pb) (lanes 2 and 6; Ac) or chloramine T

(lanes 3 and 7; Pb) (lanes 4 and 8; Ac). Lanes 5-8 were loaded with 50% fewer CPM of I125.

Figure 7 shows a photograph of a silver stained gel of a high salt elution fraction from a DEAE column (lane 5). Control preparations included pre-bleed chimpanzee low salt (lane 2) and high salt fractions (lane 3) a normal human low salt fraction (lane 6) and normal chimpanzee high salt fraction (lane 7), and high salt fraction derived from chimpanzee convalescent antibody positive fraction (lane 8), and low salt fraction purified in a commercial IgG Isolation System (Protein A, Pierce Chemical) from chimpanzee 92 convalescent plasma (lane 9). The molecular weight markers are shown in the first lane.

Figure 8 shows a photograph of a silver stained gel of pre-bleed and acute phase plasma derived from chimpanzee 450. The SDS-PAGE analysis shown in this Figure was performed on CsCl gradient fractions 15, 16 and 17 of pre-bleed (lanes 2, 3, and 4) and acute phase plasma (lanes 5, 6 and 7). The density for each respective fraction 15, 16 and 17 was determined at 1.2658, 1.2984 and 1.3212 gm/cc, respectively. Molecular weight standards were placed in lanes 1, 8 and 9.

Figure 9 shows an elution profile of complexed NANBH associated antigens from an affinity column linked to A3-1-A6 monoclonal antibody. The eluting buffer contained 0.5 M diethylamine, pH 11.5, and 0.5% sodium deoxycholate. Three pools (I, II and III) were made as indicated.

Figure 10 shows a photograph of a silver stained 14% SDS-PAGE on which affinity purified material, obtained from normal pre-bleed and NANBH acute phase plasma (chimpanzee 450) as described above, was fractionated. The gel contained the following preparations: molecular weight markers (lane 1), pre-bleed chimp 450 plasma pools I, II and III (lanes 2, 3 and 4) and pools I, II and III derived from NANBH acute phase chimp 450 plasma (lanes 5, 6 and 7). Lanes 8 and 9 were loaded with pre-bleed and acute material, respectively, derived from animal 450 by immune-complexing with convalescent antibody positive plasma from chimp 92.

Figure 11 presents an autoradiogram of a mixture of S35 methionine/cysteine labeled HCV infected chimpanzee hepatocyte proteins obtained from *in vitro* cell culture medium immunoprecipitated (IP) with pre-bleed or chronic phase antibody-positive chimpanzee plasma. Lane 1, Molecular Weight Markers; 94, 67, 43, 30, 20 and 14 KD. Lane 2, day 8 media from chimpanzee X623 immunoprecipitated with X174 chimpanzee sera prior to infection. Lane 3, day 8 media IP with X174 anti-HCV antibody positive bleed reactive to all four RIBA II antigens. Lane 4, day 8 media IP with human anti-HCV antibody positive bleed reactive to all four RIBA II antigens (patient CP). Lane 5, day 8 cell extract IP with pre-X174. Lane 6, day 8 cell extract IP with chronic X174. Lane 7, day 8 cell extract IP with CP. Lane 8, day 14 media IP with pre X174. Lane 9, day 14 media IP with chronic X174. Lane 10, day 14 media IP with CP. Lane 11, day 14 cell extract IP with pre X174. Lane 12, day

14 cell extract IP with chronic X174. Lane 13, day 14 cell extract IP with CP.

Figure 12 presents a representation of the computer graphical output of the amino sequences of HCV based on: (i) the hydrophilic values, shown in the top profile (Hopp); and (ii) the predicted hydropathic scale, illustrated in the bottom profile (Kyte).

Figure 13 presents a representation of the predicted secondary structure of a number of selected amino acid sequences derived from HCV.

Figure 14 shows an autoradiogram of HCV proteins labeled with I125 which were isolated using an affinity chromatographic column conjugated to affinity purified rabbit anti-DP3 antibody. Lane 1 contains molecular weight markers (Std). Lanes 2-4 contain sequential fractions eluted from a column loaded with an HCV infected tissue culture media and lanes 5-7 contain fractions eluted from a HBV infected tissue culture media.

Figure 15 shows the results of Western Blot analysis of gels on which concentrated HCV infected tissue culture media was fractionated (lane 1) reacted with rabbit anti-DP3 antibody followed by Protein A-$I^{125}$. Lane 2 contains $I^{125}$ labeled molecular weight markers.

Figure 16 illustrates the reactivity of HCV infected chimpanzee 174 pre-and post serum with micro-wells coated DP-3 peptide. Post sera was fractionated by Sephadex G-200 gel chromatography

and the void volume (IgM) as well as the IgG peak were also tested for anti-peptide activity by ELISA.

Figure 17 illustrates the reactivity of HCV infected chimpanzee pre- and post-HCV infection sera with micro-wells coated with DP-1 peptide.

Figure 18 presents a histogram representation of ELISA determination of five plasmas derived from the RL panel reacted with wells coated with peptide DP-3, DP-3A, DP-3B, and DP-3C.

Figure 19 presents the data from an epitope mapping experiment of peptide DP-3. Antibody positive RL plasma were pre-incubated with the following peptides: DP-3, DP-3A, DP-3B and DP-3C. The mixtures were subsequently tested for residual binding activity by ELISA using wells coated with DP-3.

Figure 20 presents the data from further epitope mapping of peptide DP-3. A dilution of HCV infected chimpanzee (No. 174) post-HCV infection serum was pre-incubated with increasing two-fold concentrations of DP-3A, DP-3B and DP-3C. The mixtures were subsequently tested for residual binding activity by ELISA using wells coated with DP-3.

Figure 21 presents the data from titration of DP-3 antibody binding reactivity by ELISA to establish an optimal concentration. Wells were coated with increasing two-fold concentrations of DP-3, quantitated as nanograms of peptide per well. After blocking, the wells were tested by

ELISA with HCV infected chimpanzee pre-and post- HCV infected sera diluted 1:100 and 1:1000. The reactivity is plotted as the ratio of the OD values recorded with the post-infection sera divided by the OD values recorded on comparable wells with pre-serum (P/N values).

Figure 22 presents histograms illustrating the optical density values of the antibody binding reactivity of each of 38 plasmas diluted 1:100 and tested by ELISA using wells coated with DP-3.

Figure 23 presents histograms illustrating the optical density values of the antibody binding reactivity of each of 82 plasmas diluted 1:100 and tested by ELISA using wells coated with DP-3.

Figure 24 presents the data from titration of antibody activity by ELISA using DP-3 coated wells. Plasmas yielding equivocal values in the 1:100 screen (see Figure 23) were diluted two-fold starting at a dilution of 1:20 and re-tested.

Figure 25 illustrates the results of experiments used to confirm, by peptide inhibition of anti-DP3 activity, the reactivity of plasma that was observed by ELISA. Plasma derived from the ALT panel were pre-incubated at the dilutions shown with increasing two-fold concentrations of DP-3 peptide. The plasmas were subsequently tested by ELISA for residual antibody activity with wells coated with DP-3.

Figure 26 presents the data from titration of six human plasmas for antibody reactivity to HCV E1

associated peptide DP-6. The plasma tested include 1) a normal uninfected individual 2) three individuals previously identified as HCV-convalescent (A115, Mayfield and Hart) and individuals from the ALT panel (ALT-1, anti-HCV negative; ALT-2, anti-HCV positive).

Figure 27 presents the data from titration of six human plasmas, described in Figure 26, for antibody reactivity to HCV E2 associated peptide, DP-15.

Figure 28 presents the data from titration of six human plasmas, described in Figure 26, for antibody reactivity to HCV E1 associated peptide, DP-16.

Figure 29 illustrates the relationships of the various DP peptides to the HCV polyprotein sequence and to the C-100 HCV-derived sequence.

Figure 30 schematically presents the antigen-inhibition assay of the present invention.

Figure 31 presents a standard curve for the HCV NS4 antigen-inhibition assay utilizing purified rabbit anti-DP3D IgG as coating reagent.

Figure 32 illustrates the quantitation of NS4 antigen concentration in HCV-infected chimpanzee sera using the standard curve shown in Figure 31.

Figure 33 presents a serological profile of sequential sera isolated from an HCV-infected chimpanzee (animal No. 196).

Figure 34 presents a serological profile of sequential sera isolated from an HCV-infected chimpanzee (animal No. 623).

Figure 35A presents a serological profile of sequential sera isolated from an HCV-infected chimpanzee (animal No. 174).

Figure 35B presents a confirmatory assay titration generated by pre-incubating 100 ng DP3D peptide panel in SFDM with increasing dilutions of an anti-HCV positive human plasma. The mixture is then tested for residual antigen reactivity measured by blocking the subsequent antigen inhibition reaction.

Figure 35C presents a standard curve for the HCV NS4 antigen inhibition assay utilizing affinity purified rabbit anti-DP3D antibody. For subsequent calculations the readings are corrected for background activity.

Figure 36 presents DP3D antigen-inhibition assay data for sequentially collected tissue culture media obtained from cultures of hepatocytes derived from an HCV-infected chimpanzee (animal No. 198).

Figure 37 presents DP3D antigen-inhibition assay data for sequentially collected tissue culture media obtained from cultures of hepatocytes derived from an HCV-infected chimpanzee (animal No. 2246).

Figure 38 presents a serological profile of sequential sera isolated from an HCV-infected human (donor No. 4811), including antigen-inhibition data

for the capsid antigen (38A and 38B), ALT data (38A) and level of HCV-directed antibodies (38B).

Figure 39 presents a serological profile of sequential sera isolated from an HCV-infected human (donor No. 4812), including antigen-inhibition data for the capsid antigen (39A and 39B), ALT data (39A) and level of HCV-directed antibodies (39B).

Figure 40 presents a serological profile of sequential sera isolated from an HCV-infected human (donor No. 4813), including antigen-inhibition data for the capsid antigen (40A and 40B), ALT data (40A) and level of HCV-directed antibodies (40B).

Figure 41 presents a standard curve for an assay designed to detect combined NS4 and capsid antigens. The wells are coated with a mixture of equal quantities of HRPO conjugated to DP3D and DP9B, respectively. For subsequent calculations the readings are corrected for background activity.

**Detailed Description of the Invention**

I. <u>The Pathology of Chimpanzees Experimentally Infected with Hepatitis C Virus.</u>

Burk et al. (1984A) infected a number of chimpanzees with human plasma that contained pedigreed NANBH virus: the non-immune (an animal which is susceptible to infection with NANBH) chimpanzee showed no indications of NANB. Liver enzymes were monitored in the serum of infected chimpanzees. Elevated liver enzyme, as indicated by alanine aminotransferase levels (ALT), rose at approximately 70 days post-infection with peak values noted 80 days after inoculation of infectious

material. At approximately 100 to 120 days the liver enzymes declined and remained relatively within the normal range. In addition, liver punch biopsies revealed liver pathology during this acute period of infection. The pathologic findings were essentially normal by 120 to 140 days post-infection.

Chimpanzee plasma, which had been obtained during the ascending limb of the above described ALT peak, was inoculated into another non-immune chimpanzee. With this second chimpanzee, essentially identical findings involving the latency period, pathology and enzyme elevations were observed (Burk et al. 1984A).

However, in a number of other NANBH (human infectious plasma) experimentally infected chimpanzees a distinctly different pattern of pathology was observed. In contrast to observations noted above in the original two chimpanzees, a long period of chronicity was observed in these other animals that lasted in excess of 600 days. This pattern included repeated ALT elevations and continued liver histopathologic changes consistent with viral hepatitis (Burk et al. 1984A). These results suggested that some, but not all, experimentally infected chimpanzees develop long-term chronic infection.

Based on the above observations, the chimpanzees whose hepatitis indicators had returned to essentially normal by 120 to 140 days post-infection were chosen for re-infection in order to generate high titer antibody to NANBH virus. These animals were intravenously inoculated with homologous, i.e., their own, acute phase plasma. After inoculation of these animals with homologous

acute phase plasma a small but definite enzyme elevation was observed. In addition, pathologic studies of punch liver biopsy material revealed a new episode of viral hepatitis concurrent with the enzyme elevations (Burk et al. 1984A). The same result was seen after repeated inoculations of these animals. The exacerbation of the disease state was seen in one animal that had been held without further challenge for 560 days beyond the acute phase infection. One animal which experienced no pathology for a period of 300 days was inoculated with acute phase homologous serum. Pathologic changes consistent with that of viral hepatitis were noted within ten days of infection. As a control, the same animals were inoculated with infectious Hepatitis B Virus (HBV): these animals had antibody to HBV before the initial NANBH inoculation. After inoculation with HBV no HBV-associated pathology was noted. Therefore, the pathology noted at approximately 500 days following inoculation of the acute NANBH homologous plasma indicated either (i) that the animals were not immune to re-infection, or (ii) that the initial infection had not been resolved (Burk et al. 1984A).

Electron microscopic examination during acute phase infection of the liver punch biopsies from the re-infected chimpanzees revealed both tubular cytoplasmic changes and the formation of intranuclear clusters of small particles measuring approximately 20 nm in diameter (Burk et al. 1981). Of greater significance was the observation of rare crystalline clusters of particles in the cytoplasm of the infected hepatocytes. The particles in these clusters were highly uniform and measured approximately 37 nanometers in diameter (Burk et al.

1981): similar particles were later observed in NANBH infected human liver tissues (Cabral et al.).

Burk et al. (1984B) observed that a select number of NANBH infected chimpanzees did not develop pathologic changes after homologous challenge with their own acute phase plasma during a late convalescent period: this late convalescent period was usually in excess of three years. It had also been observed that some NANBH human patients experienced a dramatic clinical improvement after a long-term period of chronicity: usually two to four years post-infection. These results suggested that an immune response had been mounted in the infected hosts and that a resulting antibody had eradicated the virus from their blood streams.

Based on the above observations, several sera were obtained from both long-term convalescent NANBH-infected chimpanzees and human patients. These sera were used to stain acute-phase, NANBH-infected chimpanzee liver sections. Routine staining methods, such as fluorescence, peroxidase and peroxidase/anti-peroxidase methodologies, all yielded negative results. However, distinct cytoplasmic staining was observed using the ABC staining method (Burk et al., 1984B). The ABC method employs an avidin-biotin complex (ABC) which presumably amplifies the sensitivity of immunologic tissue staining by a factor of 40- to 80-fold when compared to routine procedures such as indirect peroxidase, or Staph-A amplified peroxidase/anti-peroxidase methods. Utilizing this method, specific cytoplasmic staining was observed in approximately 10% of hepatocytes obtained from liver sections derived from an acute-phase, NANBH virus-infected chimpanzee. No staining was seen

when (i) using the same sera with normal, uninfected liver tissue or, (ii) using normal human sera as controls instead of the NANBH antibody-containing chimpanzee or human sera.

Burk et al. (1984B) investigated whether acute phase plasma contained an antigen similar or identical to the antigen detected in acute phase NANB virus-infected hepatocytes. This was accomplished by pre-incubating the above long-term convalescent anti-NANBH antibody-containing serum with acute phase chimpanzee plasma. After the incubation period the mixture was used to stain NANBH infected hepatocytes. An antigen was present in each of two acute phase chimpanzee plasma and in a chronic NANBH human sera: each of these sera blocked the staining reaction using the long-term convalescent anti-NANBH antibody-containing serum. As controls, normal human serum, normal chimpanzee serum, juvenile chimpanzee serum and pre-inoculation chimpanzee serum did not block the staining reaction. These results suggest that there is a circulating antigen during the acute and early chronic phases of NANBH that is antigenically similar to the antigen present in the NANBH virus-infected liver cells (Burk et al. 1984B).

In view of the above discussion it appears that the HCV infectious process undergoes three distinct phases as illustrated in Figure 1.

The first phase is an acute phase spanning the time between exposure to the virus and the period during which ALT elevations are observed. In the acute phase the presence of virus and virus antigen was suggested by the following:

a) plasma derived during this phase is infectious when inoculated into a non-immune chimpanzee, and

b) a unique antigen is present in the blood stream, in that, acute phase plasma blocks anti-HCV antibodies from reacting with HCV infected cells (Burk et al., 1984B).

The second phase is a long chronic phase. In 14 animals studied this phase can last in excess of three years or, more typically, in the majority of animals this phase is lifelong (Burk et al. 1984A).

In a few animals (3/14) the NANBH (HCV) infection is resolved in that the animals cannot be reinfected (Burk et al. 1984A). This phase appears to represent a true convalescent phase.

II. Identification of HCV-Associated Peptides Present in Acute Phase Sera.

In view of the above data, the protocol described in Example 1 was designed to purify virus and/or virus sub-units from acute phase chimpanzee plasma. A series of titrations were performed, as described in Example 1, mixing various quantities of acute phase chimpanzee plasma (i.e., the antigen source) with convalescent chimpanzee phase (as described above, antibody positive by tissue staining).

In order to begin to analyze the purified virus antigens monoclonal antibodies were generated using purified NANBH material from acute phase sera. A limiting factor in generating an immune response to the putative acid sucrose purified virus was the quantity of specific antigenic material (i.e., virus) which could be produced from 20 mls. of acute phase plasma for use in immunizing mice. To

circumvent this limitation, a crude semi-purified NANBH preparation was used to induce the initial immune response in mice. This semi-purified material was obtained as described in Example 2. Mice were inoculated with the semi-purified material and then, four to six months after their third intramuscular inoculation, the mice were boosted with a single intravenous (IV) booster of affinity acid sucrose purified preparation (Example 1). Fused spleen cell/murine myeloma cells were selected and screened for the production of antibodies that reacted with an acute phase NANBH plasma fraction. Two hybridomas specific to acute phase NANBH plasma were identified and designated N11.9 and A1-3.

As discussed above NANBH convalescent serum was identified that (i) was taken during a period when the chimpanzees were protected against challenge with homologous acute phase plasma, and (ii) contained antibodies which reacted specifically with a cytoplasmic antigen present in acute phase liver cell sections. In addition, these antibodies also reacted specifically with a circulating acute phase antigen as was demonstrated by blocking of the immuno-staining reaction when using infected hepatocytes. Example 3 describes the use of the chimpanzee convalescent sera in generating immune complexes with the chimpanzee acute phase plasma described above. Figures 3, 4 and 5 show autoradiograms of SDS polyacrylamide gels on which the iodinated immune complexes were resolved. A number of bands were observed in reactions with acute phase plasma which were not detected in reactions with pre-bleed chimpanzee plasma. These results suggest that there are antigens in the acute sera that are recognized by antibodies present in

the convalescent sera. Since the convalescent sera is believed to contain neutralizing antibodies, which allow the infected animal to overcome chronic infection, the antibodies potentially identify HCV viral antigens capable of generating such neutralizing antibodies.

NANBH sera were fractionated and analyzed by a variety of procedures to identify HCV specific peptides or proteins. Example 4 describes the partial purification of unique NANBH proteins using salt precipitation followed by ion exchange chromatography where the resulting proteins were radioactively labeled (Figure 6) or detected by silver staining (Figure 7). Example 4 also describes the fractionation of NANBH plasma by ultracentrifugation on CsCl gradients. The resulting proteins were examined by SDS-PAGE, the gel silver stained and a photograph of the gel is presented as Figure 8. NANBH specific proteins were also isolated using affinity gel chromatography where the affinity reagent was above-described monoclonal antibody A1-3-A6 (Example 5). A typical elution pattern from such an affinity column is presented in Figure 9. Pools resulting from the elution were then fractionated by SDS-PAGE and the proteins visualized by silver staining (Figure 10).

A number of polypeptides were observed in purified fractions produced by the above described biochemical and immunochemical protocols from acute stage plasma as compared to normal prebleed plasma. These plasma were obtained from a number of experimental HCV infected chimpanzees. The results of representative experiments are summarized in Table 1. These studies indicate that as many as 12 NANBH unique polypeptides, with molecular weights

ranging from 16 KD to 170 KD were identified (Tables 1 and 2).

Further, HCV specific polypeptides have been precipitated from *in vitro* cell culture of infected hepatocytes. A serum free media (SFM) formulation has been developed which supports the long term growth of primate hepatocytes (Lanford et al. 1989, herein incorporated by reference; co-pending, co-owned US Patent Application Serial No. 07/222,569, filed 21 July 1988, herein incorporated by reference; and co-pending, co-owned US Patent Application Serial No. 07/504,171, filed 3 April 1990, herein incorporated by reference). Briefly, the *in vitro* cell culture sustains primate hepatocytes in a serum-free medium comprising a basal cell culture medium (such as William's media E, Gibco BRL), a hepatocyte proliferogen (such as liver growth factor, Collaborative Research), serum albumin, a corticosteroid (such as hydrocortisone), one or both of somatotropin or prolactin, a growth/releasing factor, cholera toxin and ethanolamine.

Using this *in vitro* hepatocyte cell culture system, an experiment was performed to identify HCV specific polypeptides using an immunoprecipitation protocol using media plus cell extract derived from radio-labelled cultured hepatocytes, which were derived from an HCV *in vivo* infected chimpanzee liver (Example 6). After immunoprecipitation and protein fractionation on a gel, a prominent band was identified at approximately 27-28 KD which immunoprecipitated from the cell lysates on both days 8 and 14 using the human anti-HCV. This band compares favorably to a band identified in infected chimpanzee plasma (Example 5, Table 2). Similar

results were obtained using the hepatocytes from a chronically HCV-infected chimpanzee (Meula) and an anti-HCV sample from a human patient (Taffee).

The above results indicate that HCV peptides specifically associated with the acute infection phase have been identified using a variety of methods: in particular, 12 HCV unique polypeptides, with molecular weights ranging from 16 KD to 170 KD were identified (Tables 1 and 2). These polypeptides may prove useful in the development of effective anti-HCV vaccines and may also have applications in diagnostic systems, such as those described in the following sections.

III. Identification and Characterization of HCV Epitopes.

Amino acid sequence information for an HCV encoding polyprotein was obtained from a number of sources. The amino acid sequence data was analyzed by a variety of methods including hydrophilicity and hydropathy (Example 7). Figures 12A, 12B, and 12C show the results of the hydrophilicity and hydropathy analysis of the HCV polypeptide. A number of peptides were chosen based on hydrophobic epitope (i.e., HOPP +, Kyte -) The approximate locations of these peptides, relative to the HCV polyprotein sequence, are illustrated in Figures 12A, 12B, and 12C by solid lines between the Hopp and Kyte plots: each peptide was designated as DP1-6 and 8-18. For each of these peptides the predicted secondary structures are shown in Figures 13A, 13B, and 13C. The peptides derive from a number of putative domains of the polyprotein including non-structural regions 3 and 5, envelope, core, and membrane associated (Chiron, EPO 9030

2866.0). The amino acid sequences of the peptides are presented in the Sequence Listing.

Each of the 19 DP peptides listed in Table 3 was synthesized by standard procedures (Example 8). Each peptide was crosslinked to keyhole limpet hemocyanin and used to immunize rabbits to produce polyclonal antibodies. The endpoint titrations of the resulting antisera are presented in Table 4 (in Table 4, for example, 1E+5 = $1X10^5$). Generally, with the exception of DP2 in one rabbit, the antisera reacted with the homologous peptide at titers of 1:100,000 or greater.

In addition to these rabbit polyclonal antibodies, BALB/c mice were also inoculated with DP-3 and the resulting sera reacted with free DP-3 at anti-serum dilutions of 1:10,000 or greater.

The DP-3 peptide was attached to a solid support and rabbit anti-DP-3 antibodies were affinity purified using the immobilized DP-3 (Example 9). The isolated anti-DP-3 antibodies were then attached to a matrix. Culture media from HCV infected hepatocyte cells was then absorbed to the antibody matrix. The specifically absorbed material was then eluted from the column and radioactively labeled using $I^{125}$. The eluted material was then fractionated on a gel and the gel exposed to X-ray film. The resulting autoradiogram (Figure 14) showed a major band at approximately molecular weight 58 and two faint bands at approximately molecular weights 27 and 66.

Western blot analysis (Ausubel et al.) was also performed on the above culture media using the anti-DP-3 rabbit sera. Results of the Western analysis showed a major band at approximately molecular

weight 58 and a minor protein at a molecular weight of approximately 45 KD (Figure 15).

The above results suggest that DP-3, derived from the putative NS4 HCV specified subunit, is associated with at least one polypeptide having a molecular weight of approximately 58 KD. A protein of similar molecular weight was observed in acute phase NANBH plasma (Example 5, Tables 1 and 2).

The free DP peptides DP-1 to DP-7 were screened for reactivity with a pre- and post-NANBH infection chimpanzee plasma and with a number of human plasma (Example 10). The sera were also tested for their reactivity using the commercially available Ortho anti-HCV assay which utilizes the C-100 protein described above. The locations of the antigens relative to the HCV polyprotein coding sequence are presented in Figures 29A-29F. In particular, Figure 29B shows the region of overlap between the DP-3, DP-3D, and C-100 peptides.

The results of this screening are presented in Table 5. The known positive post-infection chimpanzee serum reacted strongly with DP-3 but did not appear to react with any of the other six peptides. All of the human sera that reacted strongly with DP-3 at a dilution of 1:100 also scored as positive at a dilution of 1:10 in the commercial (Ortho) assay. One serum reacted weakly with DP-1, DP-4 and DP-5. In addition, three sera, which scored as negative in the Ortho assay, reacted with DP-3. These data suggest that an immunodominant HCV epitope is associated with DP-3.

The DP peptides listed in Table 6 were then screened against a panel of sera obtained from a set of 19 human patients where the sera had been defined serologically as NANBH-positive. As suggested by

the above data, the data presented in Table 6 suggests that DP3 and the related peptide DP-3D contain a major immunodominant HCV epitope. In addition, a number of tested sera also reacted with the following core associated peptides: DP8, DP9A, DP9B, and DP10. Also, one patient responded to an NS5 epitope identified by peptide DP17.

The sensitivity of the ELISA's based on the peptides DP-3 and DP-1 was tested by titration of infected chimpanzee sera (Figures 16 and 17, respectively). A clear positive reaction was attained for DP-3 with serum dilutions down to 1:1,000 with a borderline positive noted at dilution 1:10,000. The chimpanzee serum appeared to be 10-fold more reactive toward DP-3 than DP-1.

In view of the data suggesting that DP-3 defines a strong HCV epitope, epitope mapping of the DP-3 peptide was performed (Example 11). The above data also indicated that antibody activity was detected in serum panels tested with DP-2. The peptides DP-2 and DP-3 contain six overlapping amino acid residues. Since no reactivity was observed with the DP-2 peptide, it was assumed that the six N-terminal residues of DP-3 were not associated with the immunodominant DP-3 HCV epitope. Three overlapping peptides encompassing the 18 carboxy-terminal residues of DP-3 were synthesized: DP-3A, presented as SEQ ID NO:1922-1931; DP-3B, presented as SEQ ID NO:1928-1935, and DP-3C, presented as SEQ ID NO:1933-1940. Each of these three peptides was tested for direct binding with five human with NANBH antibody positive sera previously shown to react with DP-3. The results of the direct binding study are presented in Figure 18. The major DP-3 binding

activity appeared to be associated with the peptide DP-3B.

To rule out that the differences in binding activity between the three overlapping peptides were associated with the physical interactions of each of these three preparations with the solid matrix, a series of inhibition experiments were performed. The results of inhibition studies are presented in Figure 19. All 5 plasma were inhibited by DP-3. Two of the plasma were inhibited to similar levels with DP-3B and about 17% by DP-3C. However, none of the plasmas were inhibited by DP-3A. The inability to inhibit one of these five plasma (RL19) with any of these three short peptides, may indicate that the binding activity of this antibody may at least in part be directed by the conformation associated with the large DP-3 peptide, which is not present with the shorter sequences.

HCV infected chimpanzee serum, tested for binding and inhibition using the above 4 peptides, showed a pattern of reactivity similar to human serum RL20. Figure 20 illustrates the results of testing the chimpanzee serum by performing the inhibition assay using increasing amounts of each short peptide. The data show that increasing quantities of DP-3B and DP-3C both inhibit antibody binding with DP-3. The peptide DP-3A showed only low level inhibition. These results suggest either that antibody against both DP-3B and DP-3C can be identified but that DP-3B binds antibody with higher avidity than DP-3C, or that there are different populations of antibodies present in the polyclonal chimpanzee anti-DP-3 serum which recognizes overlapping DP-3 epitopes.

IV. Examination of the DP-3-based Anti-HCV ELISA Assay.

The double-blind Alter NANBH serum panel was screened using the DP-3 based anti-HCV assay of the present invention. The screening results are presented in Table 7. The data show perfect concordance of the assay results with the known HCV sera status of the samples in the serum panel. Two sera tested with intermediate absorbency readings had been obtained from a patient shortly after resolution of an acute infection and were believed to represent an early stage of immune response.

The reaction conditions for the peptide based anti-HCV assay were optimized. The optimal concentration of DP-3 was determined by serial dilution of a DP-3 solution in a variety of buffers (Example 13). Reactivity was calculated as a ratio (P/N) of the absorbency value of HCV-positive sera treated wells divided by the absorbency value of HCV-negative sera treated wells. Figure 21 shows the results of one optimization experiment where the DP-3 peptide was diluted in bicarbonate buffer. Peak reactivity was observed at a well-coating concentration of 500 ng of the DP-3 peptide. A number of other components involved in the assay were varied in order to increase the sensitivity of the assay. One preferred well-washing buffer was phosphate buffered saline containing 0.5% "TWEEN" detergent (Example 13).

A serum panel was screened using the DP-3 based anti-HCV assay. The panel was 42 sera consisting of 21 paired serum samples where 19 samples had been obtained from a number of documented NANBH patients and 2 were normal serum samples (Figure 22). Approximately 84% of the NANBH sera scored as being

antibody positive using the DP-3 assay. The four negative control sera scored as negative (Table 8). The same serum panel was also screened using the Ortho anti-HCV assay. By the Ortho assay approximately 65% of the NANBH sera tested as positive (Table 8). All the sera that tested as positive in the Ortho assay also tested as positive in the DP-3 based assay. These data suggest that the DP-3 assay is more sensitive than the commercially available first generation Ortho anti-HCV assay.

Another panel of sera used to test the DP-3 based anti-HCV assay of the present invention consisted of blood donor plasma that had been rejected on the basis of elevated ALT values (Example 15). The results of this panel of sera screened using the DP-3 based assay are presented in Figure 23. Eight of the 82 plasmas tested gave strong positive reactions. Another 10 plasmas were scored as positive. Each of these 10 plasmas were tested in DP-3 anti-HCV assays using a series of diluted plasma. Each of these 10 plasmas yielded the linear dilution curves shown in Figures 24A and 24B. In addition, each of these 10 plasmas was tested using a cold-peptide inhibition protocol (Example 16). Dilutions of the plasma were pre-incubated with increasing amounts of the DP-3 peptide before testing for antibody binding using DP-3 coated wells. The data for the cold-peptide inhibition test are presented in Table 10. As can be seen from the data, antibody reactivity was inhibited from 50 to 95% by prior incubation in the presence of un-coupled DP-3 peptide. Next, both high and low reacting plasma (1:100) were pre-incubated with DP-3 free peptide. The data from

these assays are presented in Figures 25A and 25B. All plasmas were derived from the High ALT Panel; those which were strongly positive, were inhibited more than 50% by addition of 20 μg peptide (Figure 25A). Low reacting plasmas 435 and 553 were also inhibited by more than 50%: plasma 850 appeared to be a non-specific anti-HCV negative plasma having high background activity. These results confirm that plasmas which tested positive in the DP-3 based anti-HCV assay at low levels (0.2-0.5) are true positives.

When the same panel of sera having elevated ALT values was tested at a plasma dilution of 1:10, 18 (22%) antibody positive plasma were detected using the DP-3 based assay and 8 (9.8%) antibody plasma samples were detected using the Ortho anti-HCV test. All plasma detected as positive using the Ortho kit were strongly positive in the DP-3 based assay (Table 9).

The performance of the DP-3 based assay was further compared with that of the Ortho assay using serial dilutions of the serum panel described in Example 14 to determine relative antibody titers. These results are presented in Table 11. From these data the DP-3 assay is in excess of ten-fold more sensitive than the Ortho assay for HCV detection.

The relative sensitivity and specificity of the DP-3 based and Ortho anti-HCV assays were compared using a panel of 362 donor blood plasmas obtained from the Southwest Regional Blood Bank (Example 18). All plasmas that tested positive using the DP-3 based assay were confirmed with the above described peptide inhibition assay. All plasma that tested positive using the Ortho kit were re-tested using the commercially available RIBA test. The results

of these screens and associated plasma information are presented in Table 12. Six plasmas were recorded as anti-HCV positive in the DP3 assay, five of which were confirmed by specific antigen inhibition. Three positives were noted when tested by the Ortho assay, one of which was scored as non-specific because it did not react in a positive pattern with the RIBA confirmatory strip (Table 13). The DP-3 assay produced a positive test result of 1.4%, with one non-specific positive. The Ortho assay produced a positive test result of 0.6%, with one non-specific positive (Table 14). Accordingly, it appears that the DP-3 assay has increased sensitivity and comparable specificity relative to the Ortho assay.

V. Development of an Immunoassay for Detection of HCV NS4 Antigen.

The basic design of the immunoassay is illustrated in Figure 30. One example of the antigen detection assay is described in Example 20. In this example, a rabbit anti-DP3D-coated well and a DP3D-horse radish peroxidase (HRPO) conjugate were used in the assay. Microwells were coated with IgG derived by gel filtration from high titer rabbit anti-DP3D sera. The antibody coated wells were then incubated with the test samples, such as, DP3D peptide, HCV infected tissue culture media, human sera and chimpanzee sera. After incubation, DP3D-HRPO conjugate was added to each well. Combination of the conjugate to the solid phase antibody coat was detected by the addition of the substrate-2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS). An alternative substrate which can be used is 5-aminosalicylic acid (5AS). The presence of a

DP3D associated viral antigen was identified by color diminution due to successful inhibition of binding DP3D-HRPO.

A number of reporter labels, other than HRPO can be used in the method of the present invention, including the following: enzymatic reporter systems, such as HRPO alkaline phosphatase, β-galactosidase, and glucose oxidase (Pierce, Rockford IL); fluorochrome reporters, such as fluorescein, R-phycoerythrin, rhodamine, rhodamine 600, and "TEXAS RED" (Pierce); biotin and avidin (Pierce); radioactive labelling, such as $^{125}I$ or synthesis of antigen polypeptides containing $^{3}H$ or $^{14}C$; light emitting reporters, such as luciferase (de Wet, *et al.*); and chromophors, such as heme (Sigma, St. Louis MO). Reporter labels are conjugated to antigen peptides by appropriate standard methods in the art.

The antigen detection assay of the present invention was tested by addition of increasing quantities of free DP3D peptide. As shown in Figure 31, the addition of the DP3D peptide at a concentration of 10 μg/ml inhibited reactivity with DP3D-HRPO by 68%. A linear inhibition curve was observed over the range of 10 μg/ml to 0.001 μg/ml. One ng/ml DP3D inhibited the reaction by 8%.

A method to confirm the specificity of the antigen detection reaction is described in Example 21. This confirmation assay is based on blocking the inhibition of DP3D-HRPO binding to its cognate antibody, which usually occurs by binding of a DP3-based antigen to the same antibody. The blocking of inhibition is accomplished by addition of anti-DP3D positive sera to the antigen positive plasma before testing in the antibody coated plates. Prior

addition of rabbit anti-DP3D or HCV antibody positive human or chimpanzee plasma to different concentrations of synthetic peptides showed an increase in DP3D-HRPO binding to its cognate antibody, which corresponds to a decrease in antigen mediated inhibition of the DP3D-HRPO binding.

The concept that HCV induces antigenemia in detectable quantities during the acute phase of the HCV infectious disease process was tested by incubating plasma derived from six chimpanzees during the period when they demonstrated an elevation in their ALT values (Example 22). Acute phase plasma from four of the six animals inhibited the DP3D-HRPO reaction. Using the DP3D inhibition curve as a standard, the level of DP3 defined antigen ranged from 18-46 ng/ml (Figure 32). These values can be adjusted to reflect true antigen concentration using the molecular weight of the intact HCV protein associated with the DP3 defined epitope. Preliminary observations, discussed above, indicated that the DP3D epitope is associated with a protein with a molecular weight of 58 KD. Therefore, 100 pg/ml of DP3D would equate to approximately 5 ng/ml of P58.

A number of HCV parameters were evaluated for sequential plasma derived from chimpanzee 196, who was experimentally infected with tissue culture propagated HCV (Example 22). In this animal the first significant ALT elevation was observed on day 115 (Figure 33). Circulating HCV antigen was detectable, using the DP3D-antigen based detection assay as early as day 28 with peak levels detected on day 115. It was observed that antigen was not detectable when this animal began to mount an immune response to the DP3D associated virus subunit (day

178), as detected by use of a DP3D-peptide based anti-HCV ELISA (Materials and Methods). In this animal a positive PCR signal was first noted on day 115. Refinements of the PCR protocol yielded increased levels of detection: viral RNA can be detected on day 91 in the above chimpanzee.

To more fully illustrate the utility of this assay, sequential plasmas derived from two additional chimpanzees (animal Nos. 623 and 174) were also tested for ALT values, DP3D antigen and anti-DP3D. These profiles are shown in Figures 34 and 35. Chimpanzee 623 (Figure 34), also infected with tissue culture derived HCV, had detectable levels of antigen on day 301 through day 431. The first ALT elevation was noted on day 389. Once again the DP3D based HCV antigen assay was demonstrated as being capable of detecting one HCV marker prior to the occurrence of a significant ALT elevation.

Chimpanzee 174 (Figure 35) first developed ALT rises on day 69 and became anti-DP3D positive on day 280. Unlike the other animals, no antigen was detected in the plasma of the chimpanzee 174. Thus, chimpanzee 174 may have effectively resolved its HCV illness without experiencing the debilitation of the more usual course of events, i.e., chronic disease development. The absence of DP3D based antigen during acute illness might signify a more favorable clinical outcome in resolution of HCV infection. Chimpanzee 623 shows a delay in onset of anti-DP3D antibody response. Unlike chimpanzee 174, it is clear that chimpanzee 623 remains chronically infected with HCV, some 18 months beyond his acute disease episode (Figure 34).

A panel containing 82 human plasma samples derived from individuals with elevated ALT values and a second panel containing 228 plasmas from normal blood donors were also screened for the presence of HCV antigen using the antigen detection assay of the present invention (Example 23). Six specimens in the ALT panels gave a positive inhibition, two high level inhibition (approximately 10 ng/ml) and four weak or borderline (Table 18, Example 23). In the panel containing 228 blood donor specimens, two samples were detected yielding a high degree of inhibition.

To ascertain the specificity of these eight plasma samples each was tested using the blocking confirmatory assay described above. The confirmatory assay was performed by prior incubation of the positive reactant plasma with a 1:500 final dilution of an anti-HCV positive human plasma (see Figure 35B). A final dilution of anti-HCV positive plasma of 1:500 was used in subsequent testing. The mixture is then tested in the antigen assay and a positive is confirmed by blocking of the ability of the sample to inhibit the DP3D anti-DP3D reaction (Table 19). Of all of the strongly inhibiting samples, ALT 5, ALT 64 and the two reactive blood donor specimens were inhibited by greater than 80%. In addition, 100% inhibition was noted with one weak reacting plasma (ALT 4). The remaining three border line reactors were not blocked by the prior addition of anti-HCV antibody. Based on these results 3/82 plasma (3.7%) in the high ALT panel and 2/228 blood donor specimens (0.9%) contained HCV specific antigen. None of the sera that tested positive by the antigen detection assay of the present invention gave a positive test result with either a

commercially available HCV-antibody detection kit or an anti-DP3 antibody detection ELISA (Materials and Methods).

To increase the sensitivity of this assay, wells were coated with rabbit anti-DP3D purified by affinity chromatography utilizing "SEPHAROSE 4B" (Pharmacia, Piscataway, NJ) conjugated to DP3D. The standard curve is shown in Figure 35C. The reaction of DP3D-HRPO conjugate with affinity purified anti-DP3D coated wells can be inhibited 100% by incubation of 10 μg/ml DP3D. The end sensitivity of this assay using DP3D is 100 pg/ml.

The antigen detection method of the present invention was also used for the detection of HCV NS4 antigen in tissue culture (TC) media derived from hepatocytes infected with HCV. Hepatocyte cultures derived from acute or chronic stage HIV infected chimpanzees afforded the active replication of HCV (Jacob *et al.*, 1991). This active replication was demonstrated by the following: 1) electron microscopy showing virions in infected hepatocyte media; 2) specific immunostaining of HCV infected hepatocytes with convalescent sera; 3) ability to infect normal chimpanzees with tissue culture derived virus; and 4) by demonstration of both positive and negative strand RNA in infected hepatocyte lysates and of positive strand RNA in infected media by PCR.

A TC pool, derived from days 3-27 of media harvested at two day intervals from HCV infected hepatocytes, was tested for HCV DP3D associated NS4 antigen (Example 24). Media harvested from HCV infected hepatocytes inhibited the DP3D conjugate reaction by 54% (Table 20, Example 24). This equates to a level of approximately 1 μg/ml of NS4

viral protein associated with the DP3D epitope (quantified with reference to a DP3D synthetic peptide standard curve), which is secreted by the infected cells into the media. The putative virus present in the media was purified by centrifugation and cushioning onto a 60% sucrose gradient. Approximately 64% of the soluble antigen was lost during this purification step (Table 20).

The temporal synthesis of HCV NS4 antigen was investigated by testing sequential TC media derived at two day intervals from *in vivo* infected hepatocytes derived from a chronic HCV infected chimpanzee (animal No. 198). A biphasic synthesis of antigen was noted (Figure 36). Days 1-13 were positive, days 15-17 were negative with a second cycle of antigen noted on days 19-32 (Figure 36).

Sequential TC medias, derived from HCV infected hepatocytes from chronic chimpanzee 2246, were screened for DP3D associated HCV antigen. The results are illustrated in the attached Figure 37. As noted previously in media from hepatocytes derived from a chronically HCV infected chimp, two cycles of antigen production were observed, day 1 through 5 and days 5 through 15.

These experiments demonstrate the ability of the antigen detection assay of the present invention to detect HCV specific antigens from a number of sources.

VI. Utilization of Antigen Assay for Drug Screening for HCV.

Hepatocytes derived from Chronic HCV infected chimpanzee 2246 were treated with a number of drugs on day 3, including ribavirin and interferon (Table 21, Example 25). The cells were maintained on these

drugs and the TC media tested for HCV antigen. Ribavirin, used at two different concentrations, failed to significantly alter production of DP3D associated HCV antigen. On the other hand, interferon completely inhibited production of detectable levels of antigen, nine days after addition of the drug. In fact, addition of a 5-fold higher level of drug inhibited antigen production after two days exposure to the drug (Table 21).

These results demonstrate the usefulness of the present invention to evaluate the effects of drug treatment on HCV antigen production.

VII. Development of An Immunoassay for HCV Structural (Capsid) Antigen.

The immunoassay described above detects NS4, a non-structural HCV subunit. The function of NS4 protein is undefined. Based on the flavivirus model it is assumed that NS4 is not part of the intact virion. Therefore, detection of an antigen associated with one of the major structural proteins of the virus could be advantageous for screening plasma or infected tissue culture media.

The peptides DP9A, DP9B, DP10 and DP11 were tested (Example 26) in the antigen inhibition assay, essentially as described above: these peptides correspond to the putative capsid protein of HCV (Example 7, Table 3). Rabbit antisera was prepared for each peptide. Microtiter plate wells were coated with affinity purified antibody to each respective synthetic peptide (DP9A, DP9B, DP10 and DP11).

Sequential plasma samples derived from an HCV infected chimpanzee (No. 196) were tested for presence of detectable capsid antigen as described

above for the DP3 antigen (Example 20). The results of these inhibition assays (Table 23) demonstrated that antigen was detected with each respective peptide assay following the first significant elevation in ALT values and at least one later date. When the assays were performed with peptide DP11 the capsid antigen was detected substantially throughout the test period (Table 23).

In addition, all of the synthetic peptides presented in Table 3, excluding DP3A through DP3F, were screened against a panel of plasmas derived from blood donors who had been excluded due to elevated ALT values (Example 27). DP8, DP9A, DP9B and DP10 all reacted to at least one of eight individual plasma; none of these eight plasma reacted to DP11. This result suggests that the epitope associated with DP11 is not normally immunogenic in humans infected with HCV. Employing, in the present assay method, an antigen not immunoreactive with sera obtained from HCV-infected humans would exclude interference by capsid antibodies potentially present in a given HCV-infected human sera sample.

Rabbits immunized with DP11 produce a vigorous immune response to DP11. In view of (i) the strong immunoreactivity of DP11 in the sequential plasma samples derived from an HCV infected chimpanzee, (ii) the lack of immunoreactivity with the above-described human hepatitis panel, and (iii) the ability to generate a strong immune response against DP11 in rabbits, DP11 was pursued as a candidate for the development of a HCV capsid specific antigen immunoassay.

Given that DP11 did not appear to be immunoreactive with plasma obtained from HCV-

infected humans, the ability of detergent to facilitate detection of the DP11 antigen was investigated. Detergent treatment may strip envelope material from intact virions or cause a partial denaturation of protein, either of which may promote the detection of the capsid antigen in samples. Chimpanzee sera which were scored as positive for capsid antigen in the DP11/anti-DP11 assay were retested by dilution in 0.5% Tween 20/PBS. A level of inhibition of 23% was noted with undiluted plasma. Dilution in Tween 20/PBS yielded levels of 29% and 35% at plasma dilutions of 1:2 and 1:4, respectively. These results indicated that the addition of detergent was not detrimental to the assay method and further seemed to enhance detection of DP11. These conditions, *i.e.*, including the addition of a surfactant, appear to promote the binding of the anti-DP11 antibody to the HCV antigen DP11 derived from the HCV-infected human samples where it is not normally immunoreactive.

Example 28 describes the development of an inhibition HCV antigen assay protocol that employs DP11 antigen and which includes a detergent. The detergent used in Example 28 is "TWEEN 20." Other detergents may be useful in the practice of the present invention including other polyoxyethylene sorbitans and non-ionic surfactants. The efficacy of any given detergent and appropriate concentrations can be determined using the assay method described in Example 28.

The inhibition HCV antigen assay protocol employing DP11 antigen was used to screen three HCV seroconversion panels (4811, 4812, and 4813). These panels were derived from human donors infected with HCV via human RBC preparations. Sequential sera

samples from these panels were analyzed (Example 29) for capsid antigen DP11, ALT values, and the presence of HCV antibodies as determined using a commercial kit. The results of these three determinations are presented in Figures 38, 39 and 40.

In all three individuals low levels of DP11 associated antigen were detected early in the course of infection. In all the panels, at the time point approximately corresponding to the elevation of the ALT values, DP11 antigen became undetectable. Upon resolution of the pathology, as noted by decline of the ALT values, all three individuals again developed positive antigen reactivity. The above results suggest that DP11 provides a useful marker for early and sustained detection of infection by HCV.

The time course for the appearance of DP9B has also been investigated. DP9B is a highly immunogenic synthetic peptide (Table 3, Table 4) associated with HCV capsid protein. DP9B was used in screens against sequential tissue culture media samples harvested from hepatocytes derived from a chronic HCV infected chimpanzee. The assay design was similar to that described above for the NS4 specific test. Wells were coated with affinity purified rabbit anti-DP9B and the detection probe consisted of DP9B conjugated to HRPO. As noted above, a number of different reporter systems can be conjugated to the capsid antigen. This analysis suggested that the capsid antigen is expressed at relatively high levels on Day 1, but disappears rapidly. Using the sample samples and a DP3D probe, a biphasic pattern for production of NS4 protein was observed. These results suggest that the expression

of the two viral subunits are not dependent on each other, i.e., one antigen may be produced while the second subunit is not expressed in detectable levels. Further, experiments performed in support of the present invention suggest that the immune response to these two antigens, the structural (capsid) DP9B and non-structural (NS4) DP3D, can also be independent. Accordingly, for the detection of HCV infection, the use of combinations of peptide antigens provides another screening approach.

An assay is described in Example 30 which combines the DP3D and DP9B reagents (coating antibody and HRPO - peptides probes) to detect both NS4 and/or capsid antigen simultaneously. A inhibition curve with a mixture of the two peptides is shown in Figure 41. The sensitivity of this combined assay is equal or greater than 100 pg/ml.

The above results suggest that the antigen detection assay of the present invention is a more sensitive detector of infection than detection of anti-HCV antibodies alone.

## VIII. Utility

### A. Diagnostic Method and Kit

The antigens obtained by the methods of the present invention are advantageous for use as diagnostic agents for anti-HCV antibodies present in HCV-infected sera; particularly, the DP-3 antigens (DP-3, DP-3B, DP-3C, DP-3D) and DP11.

#### 1. Antibody Capture Assays.

As noted above, the DP-3D antigen provides an advantage over known HCV antigen reagent C-100 in that it is immunoreactive with a wider range of NANBH infected sera, particularly acute-infection

sera. Epitope mapping of the DP3 defined epitope is described in Example 20*B.

In one preferred diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound HCV antigen obtained by the methods of the present invention, e.g., the DP-3D antigen. After binding anti-HCV antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-HCV antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric or colorimetric substrate.

The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group.

In a second diagnostic configuration, known as a homogeneous assay, antibody binding to a solid support produces some change in the reaction medium which can be directly detected in the medium. Known general types of homogeneous assays proposed heretofore include (a) spin-labeled reporters, where

antibody binding to the antigen is detected by a change in reported mobility (broadening of the spin splitting peaks), (b) fluorescent reporters, where binding is detected by a change in fluorescence efficiency, (c) enzyme reporters, where antibody binding effects enzyme/substrate interactions, and (d) liposome-bound reporters, where binding leads to liposome lysis and release of encapsulated reporter. The adaptation of these methods to the protein antigen of the present invention follows conventional methods for preparing homogeneous assay reagents.

In each of the assays described above, the assay method involves reacting the serum from a test individual with the protein antigen and examining the antigen for the presence of bound antibody. The examining may involve attaching a labeled anti-human antibody to the antibody being examined (for example from acute, chronic or convalescent phase) and measuring the amount of reporter bound to the solid support, as in the first method, or may involve observing the effect of antibody binding on a homogeneous assay reagent, as in the second method.

Also forming part of the invention is an assay system or kit for carrying out the assay method just described. The kit generally includes a support with surface-bound recombinant HCV antigen (e.g., the DPD-3 antigen, as above), and a reporter-labeled anti-human antibody for detecting surface-bound anti-HCV-antigen antibody.

As discussed in Section II above, several antigens associated with acute phase sera have been identified. These antigens may prove to be useful in detecting acute HCV infection in human serum. In particular, one or more peptide antigens that are

immunoreactive with acute chimpanzee sera can be combined with the DP-3D antigen to provide a diagnostic composition capable of immunoreacting with a high percentage of both chronic and acute human HCV-positive sera. Further, peptides obtained from putative core regions of the HCV polyprotein (DP-8, DP-9A, DP-9B, and DP-10) and an NS5 epitope identified by DP-17 were shown to react with human HCV-positive sera. These peptides can be combined with each other and/or DP-3 or DP-3D for immunological detection of HCV infected sera.

2. Antigen Capture Assays.

Another diagnostic configuration involves use of the anti-HCV antibodies capable of detecting HCV specific antigens. The HCV antigens may be detected, for example, using an antigen capture assay where HCV antigens present in candidate serum samples are reacted with an HCV specific monoclonal or polyclonal antibody. The antibody is bound to a solid substrate and the antigen is then detected by a second, different labelled anti-HCV antibody. Antibodies can be prepared by standard methods, utilizing the peptides of the present invention, that are substantially free of serum proteins that may affect reactivity (e.g., affinity purification (Harlow et al.)). Alternatively, the above described antigen competition assay (Example 20: DP-3D-HRPO) can be used to detect the binding of specific antigens to an antibody.

3. Antigen Inhibition Assay.

The above-described peptide antigens can also be employed in antigen inhibition assays. For this method, the DP11 antigen provides the advantage that

its use in the assay of the present invention excludes interference by capsid antibodies potentially present in human HCV-infected plasma or sera.

In one preferred diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound anti-HCV-antigen antibody, either polyclonal or monoclonal, obtained by the methods of the present invention: for example, an anti-DP11-antigen antibody. After exposure to the test serum, the solid phase is washed and then contacted with a reporter labeled antigen containing the epitope corresponding to the surface-bound anti-HCV-antigen antibody. In the case of DP11, this contacting is conducted in the presence of a surfactant, such as "TWEEN." The level of reporter is then quantitated and the serum-antigen levels are determined based on the percent inhibition of antigen-reporter binding obtained in the presence of the antigen-containing serum, typically by comparison to a standard curve.

A number of alternative-reporter systems have been described above. In one embodiment, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric or colorimetric substrate.

The solid surface reagent in the antigen inhibition assay is prepared essentially as described above.

In each of the antigen inhibition assays described above, the assay method involves reacting the serum from a test individual with a support bound anti-HCV antibody and examining the antibody for the presence of bound antigen.

Also forming part of the invention is an assay system or kit for carrying out the assay method just

described. The kit generally includes a support with surface-bound anti-HCV antigen (e.g., the DP-3D NS-4 antigen or the DP11 capsid antigen, as described above), and a reporter-labeled cognate antigen (e.g., DP3D-HRPO and DP11-HRPO, respectively) for detecting antibody bound HCV-antigen. The more antigen, from a test sample, bound to the antibody the more inhibition of reporter-antigen binding: accordingly, lower levels of detectable reporter.

Polyclonal and monoclonal antibodies, for use in the present invention, can be prepared by standard methods (Harlow, *et al.*) utilizing the peptides of the present invention. Antibodies can also be generated by recombinant techniques (Cabilly, *et al.*; Better, *et al.*; Skerra, *et al.*). In addition to whole antibody molecules, antibody fragments retaining the immunological specificity of the whole antibody may also be used in the practice of the present invention (e.g., Fab and $F(ab')_2$ fragments of IgG (Pierce)). The antibodies can be purified by standard methods to provide antibody preparations which are substantially free of serum proteins that may affect reactivity (e.g., affinity purification (Harlow et al.)).

4. Combined Antigen-Inhibition Immunoassays.

A number of the antigenic peptides of the present invention can be used singly or in combination in the antigen-inhibition assay of the present invention. In particular, one or more peptide antigens that are immunoreactive with acute chimpanzee sera can be combined with the DP-3D antigen to provide a diagnostic composition capable of immunoreacting with a high percentage of both

chronic and acute human HCV-positive sera. Further, peptides obtained from putative capsid regions of the HCV polyprotein (DP-8, DP-9A, DP-9B, DP-10 and DP11) and an NS5 epitope identified by DP-17 were shown to react with human HCV-positive sera. Antibodies generated against these peptides can be combined with each other and/or anti-DP-3D antibodies for immunological detection of HCV infected sera.

When multiple anti-HCV antibodies are used the multiple antigen-reporter molecules can be similarly or differentially labeled. For example, microtiter plate wells (i.e., multiwell plates (Corning)) can be coated with a mixture of antibodies containing equal quantities of anti-DP3D-antigen and anti-DP9B-antigen antibodies. The probe is then a mixture of, for example, DP3D-HRPO and DP9B-HRPO. The results of this assay are then read as a single-specificity.

Alternatively, the two antigen-reporter molecules can be differentially labeled by, for example, labeling one antigen with HRPO and the second antigen with alkaline phosphatase. Another embodiment of differential labeling is the use of two fluorescent reporters having different emission wavelengths: for example, phycoerythrin (PE) and fluorescein isothiocyanate (FITC). Multi-well microtiter plates can then be scanned (Dynatech Corp., Cambridge MA) and the relative levels of antigens determined based on the emission spectra.

As above, the present invention also includes kits containing multiple antibodies and cognate antigen-reporter complexes.

B. Peptide Vaccine.

The HCV antigens identified by the methods of the present invention can be formulated for use in a HCV vaccine. The vaccine can be formulated by standard methods, for example, in a suitable diluent such as water, saline, buffered salines, complete or incomplete adjuvants, and the like. The immunogen is administered using standard techniques for antibody induction, such as by subcutaneous administration of physiologically compatible, sterile solutions containing inactivated or attenuated virus particles or antigens. An immune response producing amount of virus particles is typically administered per vaccinizing injection, typically in a volume of one milliliter or less.

In particular, the peptides that have been identified which react with convalescent sera (Example 19) are excellent candidates for vaccine compositions. Further, combining acute and chronic phase antigens identified by the present invention may provide an increased vaccine efficacy for boosting a broader range immunogenic response. Vaccines are administered at periodic intervals until a significant titer of anti-HCV antibody is detected in the serum.

C. Passive Immunoprophylaxis.

The anti-HCV antibodies of the invention can be used as a means of enhancing an anti-HCV immune response since antibody-virus complexes are recognized by macrophages and other effector cells. The antibodies can be administered in amounts similar to those used for other therapeutic administrations of antibody. For example, pooled gamma globulin is administered at 0.02-0.1 ml/lb

body weight during the early incubation of other viral diseases such as rabies, measles and hepatitis B to interfere with viral entry into cells. Thus, antibodies reactive with, for example, the DP-3D antigen can be passively administered alone in a "cocktail" with other anti-viral antibodies (for example against the peptides described in Example 19) or in conjunction with another anti-viral agent to a host infected with an HCV virus to enhance the immune response and/or the effectiveness of an antiviral drug.

The following examples illustrate, but in no way are intended to limit the present invention.

## Materials and Methods

*E. coli* DNA polymerase I (Klenow fragment) was obtained from Boehringer Mannheim Biochemicals (BMB) (Indianapolis, IN). T4 DNA ligase and T4 DNA polymerase were obtained from New England Biolabs (Beverly, MA); Nitrocellulose filters were obtained from Schleicher and Schuell (Keene, NH).

Synthetic oligonucleotide linkers and primers were prepared using commercially available automated oligonucleotide synthesizers. Alternatively, custom designed synthetic oligonucleotides may be purchased, for example, from Synthetic Genetics (San Diego, CA). cDNA synthesis kit and random priming labeling kits were obtained from Boehringer-Mannheim Biochemical (BMB, Indianapolis, IN).

Oligonucleotide sequences encoding peptides can be either synthesized directly by standard methods of oligonucleotide synthesis, or, in the case of large coding sequences, synthesized by a series of cloning steps involving a tandem array of multiple

oligonucleotide fragments corresponding to the coding sequence (Crea; Yoshio et al.; Eaton et al.). Oligonucleotide coding sequences can be expressed by standard recombinant procedures (Maniatis et al.; Ausubel et al.)

Alternatively, peptides can be synthesized directly by standard *in vitro* techniques (Applied Biosystems, Foster City CA).

Common manipulations involved in polyclonal and monoclonal antibody work, including antibody purification from sera, were performed by standard procedures (Harlow et al.). Pierce was a source of many antibody reagents.

General ELISA Protocol for Detection of Antibodies.

Polystyrene 96 well plates Immulon II (PGC) were coated with 5 ug/mL (100 $\mu$L per well) peptide in 0.1 M carb/bicarbonate buffer, pH 9.5. Plates are sealed with parafilm and stored at 4°C overnight.

Plates are aspirated and blocked with 300 uL 10% NGS and incubated at 37°C for 1 hr.

Plates were washed 5 times with PBS 0.5% "TWEEN-20".

Rabbit antisera were diluted in 0.1 M PBS, pH 7.2. The desired dilution(s) of antisera (0.1 mL) were added to each well and the plate incubated 1 hours at 37°C. The plates was then washed 5 times with PBS 0.5% "TWEEN-20".

Horseradish peroxidase (HRP) conjugated goat anti-rabbit antiserum (Cappel or other commercial source) was diluted 1/5,000 in PBS. 0.1 mL of this solution was added to each well. The plate was incubated 30 min at 37°C, then washed 5 times with PBS.

Sigma ABTS (substrate) was prepared just prior to addition to the plate.

The reagent consists of 50 mL 0.05 M citric acid, pH 4.2, 0.078 mL 30% hydrogen peroxide solution and 15 mg ABTS. 0.1 mL of the substrate was added to each well, then incubated for 30 min at room temperature. The reaction was stopped with the addition of 0.050 mL 5% SDS (w/v). The relative absorbance is determined at 410 nm.

## EXAMPLE 1

## PURIFICATION OF NANBH ANTIGEN(S) IMMUNE COMPLEXES AND FRACTIONATION USING ACID SUCROSE GRADIENTS

This example describes a typical immune-complexing experiment.

Twenty mls. of convalescent antibody positive heat inactivated serum (obtained from chimpanzee 92) were mixed with 40 mls. of acute phase heat inactivated serum (chimpanzee 450). The mixture was incubated with shaking at 37°C for two hours. The mixture was then incubated for eight days at 4°C. The mixture was then centrifuged at 5000 RPM for 30 minutes and the pellet washed in cold 0.15 M NaCl using a similar centrifugation step. The resulting pellet was resuspended in 1 ml cold 1.0 M acetic acid, pH 2.3, and the acidified mixture was brought to a sucrose concentration of 5% by addition of 50 mg of sucrose. This solution was subsequently incubated for three hours at 4°C with stirring. The pH was then adjusted to 3.2 using 0.1 N NaOH. The mixture was centrifuged again at 5,000 RPM at 4°C for 30 minutes and the precipitate discarded. The resulting supernatant was layered onto a 9 mls. continuous 10% to 50% sucrose gradient: the sucrose

in the gradient was formulated in 0.1 M acetic acid, pH 3.2.

The gradient was centrifuged for 18 hours (+5°C) at 21,500 RPM in a Beckman SW41 Ti rotor. At end of the centrifugation the gradient was collected in 1 ml. fractions. Each fraction was titrated with 0.1 N NaOH to a neutral pH (pH 6.8-7.2). A representative elution profile is shown in Figure 2. The markers were prepared as followed: purified human IgG, IgM, and hepatitis B surface antigen were idinated by Chloramine-T oxidation were used to identify fractions: IgG was found in fractions 10 and Il; IgM in fractions 7 and 8; and HBsAg in fraction 1 and 2. Similar elution profiles were observed using immune complexes made with chimpanzee plasmas from NANBH acute phases and convalescent phases obtained from several individual animals.

## EXAMPLE 2

## MONOCLONAL ANTIBODY PRODUCTION USING PURIFIED NANBH MATERIAL

Fibrin was removed from acute phase plasma by the addition of $MgCl_2$. The globulin portion of the above defibrinated plasma was precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$ at 4°C. The resulting precipitate was washed with a 50% solution of saturated $(NH_4)_2SO_4$ and resuspended in phosphate buffered saline, pH 7.2 (PBS). The resuspended globulin fraction was dialyzed and fractionated by gel filtration using a "SEPHADEX G-200" matrix, equilibrated in PBS, pH 7.2. The void volume was pooled and concentrated by ultra-filtration through an Amicon PM-10 membrane. The concentrated void pooled material was used to immunize mice.

Four to six months after their third intramuscular inoculation of the "SEPHADEX G-200" purified material, the mice were boosted with a single intravenous (IV) booster of affinity acid sucrose purified preparation (fractions 1-5) (Example 1). Spleen cells from these mice were obtained three-four days after the IV booster inoculation. These spleen cells were fused with murine myeloma cells. The fused cell products were plated in HAT media as previously described (Kohler et al. 1976; Kennedy et al. 1983).

All wells which replicated hybrid cells were screened against the following antigens:

1) "SEPHADEX G-200" purified salt-precipitated material derived from acute phase NANBH chimpanzee plasma;

2) "SEPHADEX G-200" purified material from pre-plasma obtained from the same chimpanzee before experimental inoculation of the animal with known human NANBH plasma;

3) normal human serum albumin; and

4) normal human immunoglobulin G.

Microfilter wells were coated with antigen as listed above, in carbonate/bicarbonate buffer, pH 9.5 and coated overnight at 4°C. Aspiration of wells was followed by post coating with 2% human serum in PBS, pH 7.5 for 30 minutes at room temperature. Supernatant fluid samples were then incubated in the coated wells for 1 hour at 37°C, then aspirated and the wells washed three times with dI water. A biotinylated anti-mouse heavy chain IgG (Bector Labs, Burlingame, CA) in 2% human serum, PBS, pH 7.5 was incubated for 1 hour at 37°C. The wells were then aspirated and washed three times with dI water. An avidin-biotin-alkaline

phosphatase conjugate in PBS-"TWEEN" was incubated for 15 minutes at room temperature, followed by a 5X wash with PBS, addition of substrate (P-nitrophenyl phosphate, DNPP) for 30 min at 45°C, inactivation of the reaction with 1N NaOH, and final reading on a microtiter plate reader (Dynatech 600, Alexandria, VA) at 405 nm wavelength.

Wells that showed a positive reaction with the preparation derived from acute phase NANBH plasma fraction and failed to react with the other three materials were subcloned and expanded. Two hybridomas with this specificity were identified and designated N11.9 and A1-3.

## EXAMPLE 3

## FRACTIONATION OF NANBH SPECIFIC PROTEINS BY IMMUNE COMPLEXING OF NANBH PLASMA AND EVALUATION BY SDS-PAGE

An aliquot of chimpanzee acute phase plasma was immune complexed with convalescent antibody positive plasma as described in Example 1 above. The putative antigens were fractionated in acid sucrose gradient ultracentrifugation (Example 1, Figure 2), iodinated with $I^{125}$ using a chloramine-T procedure (Greenwood et al.) ($^{125}I$ through Amersham). The iodinated proteins were analyzed by SDS-polyacrylamide gel electrophoresis using 14% polyacrylamide gels. The gels were then exposed to "X-OMAT" X-ray film (Kodak, Rochester NY).

The resulting autoradiogram revealed the presence of several unique protein bands in iodinated material derived from acute phase plasma obtained from two different chimpanzees, Nos. 450 and 92, that had been complexed with convalescent antibody (chimpanzee No. 159). The autoradiogram is

shown in Figure 3. Seven bands were noted in the acute phase plasma, which were not detected in pre-bleed chimp 341 plasma that was complexed to the same convalescent antibody positive plasma. In Figure 3 the lanes are as follows: lane 1, 450 NANB vs. 159; lane 2, 92 NANB vs. 159; lane 3, 450 pre-bleed vs. 92; lane 4, same as lane 2; and lane 5, same as lane 3. These bands suggest proteins of 16 kilodaltons (KD), 20 KD, 27 KD, 29 KD, 40 KD, 48 KD, 58 KD and 104 KD.

Similar experiments were performed with acute phase plasma derived from a number of animals complexed to chimpanzee 92 antibody. In addition to the bands formed from the acute phase plasma of chimpanzee 450 and 92 (above) similar unique bands were also identified for animals 341 (Figure 4) and 325 (Figure 5), using chimp 92 convalescent antibody (Figs 4 and 5) or a human convalescent antibody (Fig 5, Milton). In Figure 4 the lane contents are as follows: lane 1, 341 pre-bleed vs. 92 antibody; lane 2, 341 NANB (day 63) vs. 92 antibody; 341 NANB (day 135) vs. 92 antibody; and 341 NANB (day 139) vs. 92 antibody. In Figure 5 the lane contents are as follows: lane 1, 325 pre-bleed vs. 92 antibody; lane 2, 325 NANB (day 57) vs. 92 antibody; lane 3, 325 NANB (day 106) vs. 92 antibody; lane 4, 325 NANB (day 112) vs. 92 antibody; lane 5, 325 NANB (day 57) vs. Minton, a human convalescent antibody; lane 6, 325 NANB pre-bleed vs. 92 antibody.

Thus, Figures 4 and 5 illustrate unique bands from NANB plasma from several acute phase chimpanzees which were immune complexed purified with several different sources of convalescent antibody, both chimp and human in origin.

Several proteins were noted in at least three of these five comparisons including p27, p29, p36, p40 and p45. Other proteins either were less abundant or did not iodinate as efficiently, including p16, p20, p58, p104 and p116.

## EXAMPLE 4

## SDS-PAGE ANALYSIS OF NANBH PREPARATIONS FRACTIONATED BY ALTERNATE PROTOCOLS

Partial purification of NANBH unique proteins was accomplished using a salt precipitation followed by ion exchange chromatography. The globulin fraction of both pre-bleed and acute phase plasmas were precipitated by the addition of equal volumes of saturated $(NH_4)_2SO_4$ at 4°C. Precipitated proteins were washed by resuspension in 50% saturated $(NH_4)_2SO_4$ at 4°C and repelleted by slow speed centrifugation. The precipitate was resuspended in 0.01 M sodium phosphate buffer, pH 6.4, and dialyzed against the same buffer overnight.

The proteins were placed on "DEAE-SEPHAROSE" columns, which were equilibrated in 0.01 M sodium phosphate, pH 6.4. After elution of a major peak (corresponding IgG), the absorbed proteins (corresponding IgG) were eluted with a high salt buffer (1.0 M NaCl, 0.01 M sodium phosphate, pH 6.4). The resulting high salt fraction was radio-labelled with $I^{125}$ using two different methods: chloramine-T oxidation (Greenwood et al. 1963), or $I^{125}$ uptake in the presence of enzymobeads (Pierce Chemical, Rockford, IL). This comparison was to insure than chemical damage to the protein was minimized. The resulting solubilized labeled material was fractionated using 14% polyacrylamide

gel electrophoresis under reducing conditions. The gel was then exposed to X-ray film.

Figure 6 illustrates such an autoradiogram where the lane contents were as follows: lane 1, 450 pre-bleed, enzymobead-label; lane 2, 450 acute, enzymobead-label; lane 3, 450 pre-bleed, chloramine-T-label; lane 4, 450 acute, chloramine-T-label; lanes 5-8 correspond to lanes 1-4 loaded at half the cpms. Two unique bands were noted in material derived from acute phase 450 plasma with estimated molecular weights of 27KD and 45KD.

Similar unlabeled material was fractionated by SDS-PAGE and silver stained (Figure 7). The lane contents of Figure 7 are as follows: lane 1, molecular weight standards; lane 2, 450, pre-bleed, IgG; lane 3, 450, pre-bleed, IgM; lane 4, 450 NANB, IgG; lane 5, 450, NANB, IgM; lane 6, human IgM, purified as above as a negative human control; lane 7, purified as above as a negative human control; lane 8, chimp 92 IgG; and lane 9, chimp 92 IgM. A number of unique bands were observed in the stained gel as indicated by dots next to lane 5 of Figure 7. The molecular weights of these unique bands were estimated as 21 KD, 27 KD, 40 KD, 44 KD and 116 KD. These bands were not observed in a number of control preparations which were also separated on this gel, including low and high salt eluted fractions obtained from normal chimpanzee and human serum (see lanes 6-9, Figure 7).

Chimpanzee 450 pre-bleed and acute phase plasma were next fractionated by isopycnic banding via ultracentrifugation on 20-40% CsCl gradients. 0.5 ml fractions were collected from the top of the gradient. Fractions 15, 16, and 17 obtained from prebleed and acute plasma were separated by SDS-PAGE

on a 14% gel. The gel was then silver stained and a photo of the gel is presented as Figure 8. The lane contents are as follows: lanes 2-4, 450 pre-bleed, fractions 15, 16, and 17, respectively; lanes 5-7, 450 NANB acute phase, fractions 15, 16, and 17, respectively; and lanes 1, 8 and 9 contained molecular weight standards.. One unique band was noted by silver staining (lane 7, Figure 8) which had a molecular weight of approximately 27K. The density of this CsCl fraction was determined to be 1.3212 gm/cc.

## EXAMPLE 5

## SDS-PAGE ANALYSIS OF NANBH PREPARATIONS ISOLATED BY AFFINITY GEL CHROMATOGRAPHY

As described above (Example 2), two monoclonal antibodies have been generated to NANBH chimpanzee plasma. One of these monoclonal antibodies (A1-3-A6) was covalently linked to "SEPHAROSE 4B" (Pharmacia, Piscataway NJ) using CNBr for cross-linking. Two identical affinity columns were used to fractionate partially purified preparations from a pre-bleed and an acute phase plasma derived from chimpanzee 450.

The partial purification was carried out by 50% salt $(NH_4)_2SO_4$ precipitation followed by gel filtration chromatography on "SEPHADEX G-200" (Pharmacia, Piscataway NJ) (as described in Example 2). The partially purified preparations were separately loaded on each of the two identical columns containing the monoclonal antibody (A1-3-A6). The columns were washed with 50 ml of each of the following:

1) 0.5 M NaCl, 0.05 M Tris-Hcl, pH 8.2, 0.001 M EDTA and 0.5% "NONIDET P-40" (Sigma, St. Louis, MO);

2) 0.15 M NaCl, 0.05 M Tris-HCI, pH 8.2, 0.5%, "NONIDET P40" and 0.1% SDS.

The columns were then washed with 100 mls. of 0.15 M NaCl and 0.5% sodium deoxycholate.

After the majority of non-specific protein had been washed through the column and the $A_{280}$ OD readings approached baseline, complexed antigen was eluted from the columns using a buffer containing 0.5 M diethylamine, pH 11.5, and 0.5% sodium deoxycholate. A typical elution pattern is shown in Figure 9.

Three pools were formed from the collected fractions (illustrated in Figure 9). Each pool was then fractionated by SDS-PAGE on 14% gels. The proteins were visualized by silver staining. The silver staining pattern is shown in the photograph presented as Figure 10. The lane contents in Figure 10 are as follows: lane 1, molecular weight standards; lanes 2-4, affinity purified 450 pre-bleed, pools I, II and III, respectively; lanes 5-7, affinity purified 450 acute, pools I, II, and III, respectively; lane 8, 450 pre-bleed vs. 92 antibody; and lane 9 450 NANB vs. 92 antibody IgG. A number of unique protein bands can be seen in the lanes corresponding to pools I and II derived from acute phase plasma that are not present in similar pools derived from pre-bleed normal chimpanzee 450 plasma. These unique proteins include bands with approximate molecular weights of 27 KD, 30 KD, 40 KD, 45 KD, 58 KD, 88 KD, 116 KD and 170 KD.

These above results suggest that:

1) monoclonal antibody A3-1-A6 may not be monoclonal;

2) that the antibody may combine to another antibody which complexes to intact virions; or

3) that the A3-1-A6 antibody has specificity for a conformation-dependent virus epitope which is cryptic unless the virus has complexed to anti-viral antibody. Recent data indicate that one of the latter two explanations is the most tenable.

Table 1 provides a summary of the unique NANBH polypeptides identified by the above-methods in acute phase plasma derived from HCV infected chimpanzees.

Table 1

| HCV Unique Polypeptide | IMMUNE-COMPLEX I-125* | | | | | DEAE-High Sal | | Affinity | CsCl |
|---|---|---|---|---|---|---|---|---|---|
| | 340** VS 92 | 325 VS 92 | 92 VS 92 | 450 VS 92 | 450 VS 92 | 450 Ag*** | 450 I-125 | 450 AG | 450 Ag |
| P16 | 16 | | | | 16 | | | | |
| P20 | | | 21 | | | 21 | | | |
| P27 | 26 | 27 | | | 26 | 27 | 27 | 27 | 27 |
| P29 | 29 | | 28 | | 29 | | | 30 | |
| P36 | | 34 | | 36 | 38 | | | | |
| P40 | 40 | 39 | 39 | 41 | 40 | 40 | | 40 | |
| P45 | 48 | | 42 | 45 | 48 | 44 | 45 | 45 | |
| P58 | 58 | | | | | | | 58 | |
| P88 | | | | | | | | 88 | |
| P104 | 104 | | | | 104 | | | | |
| P116 | | | | | | 116 | | 116 | |
| P170 | | | | | | | | 170 | |

*Proteins labeled with I-125 and visualized by autoradiograms.

**Chimpanzee designations.

***Proteins visualized by silver (Ag) staining.

Table 2 provides a summary of the molecular weight ranges of the unique polypeptides detected in acute phase NANBH chimpanzee plasma.

Table 2

| Polypeptide | Mol Wt Range |
|---|---|
| p16 | 15-19 KD |
| p20 | 20-22 KD |
| p27 | 26-29 KD |
| p29 | 28-30 KD |
| p36 | 34-36 KD |
| p40 | 38-42 KD |
| p45 | 44-45 KD |
| p58 | 58-59 KD |
| p88 | 88 KD |
| p100 | 100-104 KD |
| p116 | 115-117 KD |
| p170 | 170 KD |

## EXAMPLE 6

## IMMUNOPRECIPITATION OF HCV SPECIFIC POLYPEPTIDES FROM *IN-VITRO* CULTIVATED HEPATOCYTES

Hepatocytes were isolated from an HCV infected chimpanzee (X623), during the acute phase of HCV infection. The cells were isolated and grown in SFM using our standard methods (Jacob, et al., 1990A and B). On days 8 and 14 post-seeding a 60 mm plate was labeled for 20 hr in 2.5 ml SFM containing 500 μCi of $^{35}S$ methionine/cysteine mixture (New England Nuclear, "EXPRE $S^{35}S^{35}$").

The media were clarified, adjusted to contain 1% NP40, and the cell monolayer was washed three times with PBS and extracted with EB (Tris HCl 50

mM, pH 9.0, 100 mM NaCl, 1% NP40). The media and cell extracts were divided into three equal aliquots and immunoprecipitated using antibodies (Pharmacia, Piscataway, NJ) bound to protein A agarose. The protein A agarose-antibody beads were incubated with the samples overnight with shaking at 4°C. No buffer in media cell extracts were in EB (TRIS HCl 50 mM, pH 9, 100 mM NaCL, 1% NP 40). The beads were washed three times in EB and bound proteins were eluted with standard SDS-PAGE sample loading buffers.

The resulting immunoprecipitated proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel. The gels were then exposed to X-ray film. A photograph of the resulting autoradiogram is presented as Figure 11. The lane contents in Figure 11 were as follows: lane 1, molecular weight standards; lane 2, pre 174, media $S^{35}$, day 8; lane 3, acute 174, media $S^{35}$, day 8; lane 4, HCV positive human, media $S^{35}$, day 8; lane 5, pre 174, cell Lysate $S^{35}$ day; lane 6, acute 174, cell Lysate $S^{35}$ day; lane 7, HCV positive human, cell Lysate $S^{35}$ day; lane 8, pre 174, media $S^{35}$, day 14; lane 9, acute 17, media $S^{35}$, day 14; lane 10, HCV positive human, media $S^{35}$, day 14; lane 11, pre 174, cell Lysate $S^{35}$ day 14; lane 12, acute 174, cell Lysate $S^{35}$, day 14; lane 13, HCV positive human, cell Lysate $S^{35}$, day 14. The prominent band at approximately 27-28 KD immunoprecipitated from the cell lysates on both days 8 and 14 using the human anti-HCV. This band compares favorably to a band identified in infected chimpanzee plasma (Example 5, Table 2). Similar results were obtained using the hepatocytes from a chronically HCV-infected

chimpanzee (Beula) and an anti-HCV sample from a human patient (Taffee).

EXAMPLE 7

COMPUTER ANALYSIS OF AMINO ACID SEQUENCES DERIVED FROM THE HCV CODING SEQUENCE

This example describes the computer analysis of amino acid sequences derived from HCV nucleic acid coding sequences that examines, hydropathy, hydrophilic regions, and secondary structure.

The amino acid sequences information was obtained from the following sources: published European Patent Applications Nos. 88/310922.5 and 90/302866.0 -- for the 3′ end of the HCV nucleotide sequence; Okamoto et al. (1990) -- for the 5′ end of the HCV nucleotide sequence; and the nucleotide sequence published by Jacob et al. (1990B). These sequences were analyzed as follows: for prediction of hydrophilicity using the algorithms of Pauletti et al (1985) and Hopp-Wodds (1981); for prediction of hydropathy, the Kyte-Doolittle (1982) algorithm; to predict secondary structure the Chou-Fasman (1974) algorithm.

The hydrophilicity (Hopp) and hydropathy (Kyte) profiles for the 5′ and 3′ sequences are shown in Figures 12A, 12B, and 12C. From these analyses 17 peptide sequences were selected for further analysis. The predicted secondary structure of these 17 peptide sequences were analyzed for secondary structure by the Chou-Fasman algorithm. The resulting secondary structure profiles are shown in Figures 13A, 13B, and 13C.

Based on the putative virus protein domains of the encoded HCV polyprotein the amino acid designations relative to the polyprotein sequence

and the corresponding putative domains are also presented in Table 3. The following abbreviations are used for the polyprotein designations: NS, non-structural region; E, envelope; C, core; and M, matrix spanning.

Table 3

| Peptide Designation | Amino Acid Residue No. | Sequence Listing No. | Putative HCV Domain |
|---|---|---|---|
| DP1 | 1694-1717 | 1 | NS4 |
| DP2 | 1902-1922 | 2 | NS4 |
| DP3 | 1917-1940 | 3 | NS4 |
| DP3A | 1922-1937 | 4 | NS4 |
| DP3B | 1928-1935 | 5 | NS4 |
| DP3C | 1933-1940 | 6 | NS4 |
| DP3D | 1922-1945 | 7 | NS4 |
| DP3E | 1928-1951 | 24 | NS4 |
| DP3F | 1932-1955 | 25 | NS4 |
| DP4 | 1476-1493 | 8 | NS3 |
| DP5 | 1488-1506 | 9 | NS3 |
| DP6 | 641-660 | 10 | E2 |
| DP7 (F) | CONSERVED Flavivirus SEQUENCE, HCV UNRELATED | | |
| DP8 | 1-21 | 12 | C |
| DP9A | 35-55 | 13 | C |
| DP9B | 51-75 | 14 | C |
| DP10 | 98-121 | 15 | C |
| DP11 | 146-169 | 16 | C |
| DP12 | 233-251 | 17 | E1 |
| DP13 | 291-317 | 18 | E1 AND M |
| DP14 | 439-466 | 19 | E2 |
| DP15 | 467-493 | 20 | E2 |
| DP16 | 185-210 | 21 | E1 |
| DP17 | 2255-2275 | 22 | NS5 |
| DP18 | 2232-2255 | 23 | NS5 |

The amino acid sequences (SEQ ID NO:) corresponding to the 25 peptides are presented in the Sequence Listing. DP3A through DP3F are derived essentially from the DP3 polypeptide. DP7 is a non-HCV control peptide, the sequence of which is derived from a conserved Flavivirus epitope. DP3E

and DP3F were modified to contain N-terminal amino acid C and K residues to facilitate chemical manipulations.

## EXAMPLE 8

### SYNTHESIS OF SYNTHETIC HCV PEPTIDES

This example describes the synthesis of synthetic peptides corresponding to DP-1 through DP-7 and production of rabbit antibodies against the synthetic peptides.

The peptides DP-1 through DP-7 were synthesized by standard F-MOC procedures.

Each peptide was conjugated to keyhole limpet hemocyanin (KLH) (Pierce, Rockford IL) by two different methods: (i) the glutaraldehyde technique that links through epsilon-amino acid groups on lysine residues, or other free amino groups (Pierce); and (ii) a two-step procedure using m-maleimidobenzoyl sulfosuccinimide ester (sulfo-MBS) to link through free disulfide linkages (Pierce). For peptides that lacked any free epsilon-amino groups or sulfhydryl groups, a cysteine residue or a lysine residue was added to the N-terminus of the peptide. Further, a tyrosine residue was added to those peptides lacking tyrosine in order to facilitate labeling the peptides with radioactive iodine ($I^{125}$ or $I^{131}$).

For testing of polyclonal and/or monoclonal antibodies raised against these peptides, the peptides were linked to bovine serum albumin (BSA) using N-succinimidyl-3-(2-pyridyldithio) propionic (SPDP) as a disulfide cross-linker (Pierce Chemical).

For each peptide a mixture of cross-linked KLH forms (sulfo-MBS and glutaraldehyde cross-linked)

was emulsified with Freund's complete adjuvant (CFA) (Sigma). Each mixture was then used immunize rabbits. The rabbits were subsequently boosted with the same immunogen peptide/protein carrier mixture on day 0, the immunogen mixture in CFA at 3 weeks, and with immunogen in Incomplete Freund's Adjuvant (Sigma) at 6 weeks: each booster was via intramuscular injection. Bleedings were obtained at week 9 for each immunogen DP-1 to DP-7.

The DP-8 to DP-18 peptides and DP-3D peptide were prepared in a similar manner to that described above. Rabbits were boosted at the primary inoculation on days 14 and 28 and bled on day 38.

The resulting rabbit antisera were titrated by ELISA using microwells coated with free peptide (Harlow et al.). The end point titers of rabbit antisera prepared to designated HCV synthetic peptides are shown in Table 4.

Table 4

| Virus Subunit | Peptide Designation | Amino Acid Sequence | Anti-Sera Dilution Yielding > 0.4 O.D. Rab #1 | Rab #2 |
|---|---|---|---|---|
| Core | DP8 | 1-21 | 1E + 05 | 1E + 06 |
| Core | DP9A | 35-55 | 1E + 04 | 1E + 04 |
| Core | DP9B | 51-75 | 1E + 06 | 1E + 06 |
| Core | DP10 | 98-121 | 1E + 04 | 1E + 04 |
| Core | DP11 | 146-169 | 1E + 04 | 1E + 06 |
| E-1 | DP16 | 185-210 | 1E + 05 | 1E + 05 |
| E-1 | DP12 | 233-251 | 1E + 06 | 1E + 06 |
| E-1 | DP13 | 291-317 | 1E + 04 | 1E + 07 |
| E-2 | DP14 | 439-466 | 1E + 06 | 1E + 06 |
| E-2 | DP15 | 467-493 | 1E + 06 | 1E + 08 |
| E-2 | DP6 | 641-660 | 1E + 08 | 1E + 07 |

| Virus Subunit | Peptide Designation | Amino Acid Sequence | Anti-Sera Dilution* Yielding > 0.4 O.D. Rab #1 | Rab #2 |
|---|---|---|---|---|
| NS3 | DP4 | 1476-1493 | 1E + 06 | 1E + 06 |
| NS3 | DP5 | 1488-1506 | 1E + 06 | 1E + 07 |
| NS4 | DP1 | 1694-1717 | 1E + 05 | 1E + 08 |
| NS4 | DP2 | 1902-1926 | 1E + 03 | 1E + 03 |
| NS4 | DP3 | 1917-1940 | 1E + 04 | 1E + 07 |
| NS4 | DP3D | 1922-1945 | 1E + 06 | 1E + 07 |
| NS5 | DP17 | 2255-2275 | 1E + 06 | 1E + 06 |
| NS5 | DP18 | 2232-2255 | ND | ND |

*Each anti-sera tested in 10-fold dilutions by ELISA against unconjugated homologous synthetic peptide.

All antisera reacted specifically with each respective peptide at titers of 1:100,000 or greater on essentially all test bleed dates. The only exception was peptide DP-2 which elicited only low levels of antibody in one of two rabbits immunized.

BALB/c mice were also immunized with DP-3 linked to KLH. After three inoculations, the mice anti-peptide sera were shown to reacted with wells coated with free DP-3 at anti-serum dilutions of 1:10,000 or greater.

## EXAMPLE 9

## IDENTIFICATION OF POLYPEPTIDES IN HCV INFECTED HEPATOCYTE TISSUE CULTURE MEDIA

The following experiments were performed to determine the size of the HCV polypeptide associated

with the epitope defined by the DP-3 synthetic peptide.

A. Affinity Purification.

Rabbit anti-DP-3 antibody was purified using DP-3 peptide affinity chromatography (Pierce AminoPure AgAb; Pierce Amino Link Gel Running Buffer, PBS elution, 0.1 M Lysine, pH 3.2).

The affinity purified antibody was used to construct an anti-DP-3 matrix (Pierce Amino Link Gel Running Buffer, PBS elution, 0.1 M Lysine, pH 3.2).

Hepatocyte cells from chimpanzee No. 198 were isolated and grown in SFM by standard methods (Example 6). Two ml. of the HCV infected tissue culture media pool derived from these *in vitro* cultured cells were harvested on days 3, 11, 22, 25, 27 and 29. Each sample of tissue culture medium was absorbed to the anti-DP-3 column, washed with neutral buffer (0.01 M PBS) and eluted using an HCl glycine buffer (0.1 M), pH 3.2.

The eluted material was labeled with $I^{125}$ using chloramine-T and fractionated by SDS-PAGE on a 12% gel. The gel was exposed to X-ray film. The resulting autoradiogram is presented as Figure 14. The lane contents of Figure 14 are as follows: lane 1, molecular weight standards; lane 2, HCV T.C., Fx 3; lane 3, HBV T.C., Fx 3; lane 4, HCV T.C., Fx 4; lane 5, HBV T.C., Fx 4; lane 6, HCV T.C., Fx 5; lane 7, HBV T.C., Fx 5. In lane 2 a major band at approximately molecular weight 58 (p58) is evident in addition to two faint bands at approximately molecular weights 27 (p27) and 66 (p66).

B. Western Blot Analysis.

Six ml. of the above culture media (day 25), derived from *in vitro* culturing of HCV infected hepatocytes derived from chimpanzee 198, was concentrated to 50 ml. using a "CENTRICON-10" micro-concentrator (Amicon). The concentrated media was brought to a total volume of 400 µl by addition of SDS-ME disruption buffer and 100 µl was loaded into each of four lanes of a 12% reducing acrylamide gel (Ausubel et al.). Protein was transferred (Ausubel et al.) from the gel to nitrocellulose membrane (Schleicher & Schuell). The portion of the membrane corresponding to two lanes was incubated with 1:50 dilution of normal rabbit serum (pre-bleed from rabbit anti-DP3) and the remaining portion (two lanes) of the membrane was incubated with a 1:50 dilution of rabbit anti-DP3 serum. The membranes were incubated, washed and subsequently incubated with $I^{125}$-labeled protein-A (New England Nuclear). Excess protein-A was removed from the membranes (Ausubel et al.; Harlow et al.) and the membranes exposed to X-ray film.

The autoradiogram of the pre-bleed treated membrane showed no bands. The autoradiogram of the rabbit anti-DP-3 serum is presented as Figure 15. The lane contents of Figure 15 are as follows: lane 1, Rabbit anti-DP3; lane 2, markers. Lane 1 shows a major p58 band with a minor activity with protein of a molecular weight of approximately 45 KD (Figure 15).

The above observations suggest that DP-3, which appears to be associated with the putative NS4 HCV specified subunit, is associated with at least one polypeptide having a molecular weight of approximately 58 KD similar to a protein observed in

acute phase NANBH plasma (Example 5, Tables 1 and 2).

EXAMPLE 10

SCREENING OF A PANEL OF SYNTHETIC PEPTIDES WITH SERA OBTAINED FROM AN HCV INFECTED CHIMPANZEE

The reactivity of free peptides DP-1, DP-2, DP-3, DP-4, DP-5, DP-6 and DP-7 with anti-HCV sera was tested by ELISA using pre-immune chimpanzee serum (Chimp 174) and serum obtained 280 days after experimental infection of chimpanzee 174 (post) with a known human derived NANBH (Hutchinson) plasma. In addition, 10 human plasma, which had been pretested by the Ortho anti-HCV assay (Raritan, NJ), were tested against this panel of peptides.

Each sera was tested at 1:10 and 1:100 dilutions. The results of 1:100 serum dilutions for screening chimpanzee and human sera against the panel of DP synthetic peptides are summarized in Table 5: for comparison, the results of the same sera tested at a dilution of 1:10 with the Ortho anti-HCV assay are included.

Table 5

Serum Reactivity* (tested at 1:100)

| Serum I.D. | PEPTIDE DP-1 | DP-2 | DP-3 | DP-4 | DP-5 | DP-6 | DP-7 | ORTHO** Assay |
|---|---|---|---|---|---|---|---|---|
| **Chimpanzee** | | | | | | | | |
| 174-pre | - | - | - | - | - | - | - | - |
| 174-post | - | - | +++ | - | - | - | - | + |
| **Human** | | | | | | | | |
| 447 | - | - | +++ | - | - | - | - | + |
| 435 | - | - | + | - | - | - | - | - |
| 553 | - | - | + | - | - | - | - | - |
| 767 | - | - | +++ | - | - | - | - | + |
| 2 | ++ | - | +++ | + | + | - | - | + |
| 16 | - | - | +++ | - | - | - | - | + |
| 18 | - | - | +++ | - | - | - | - | + |
| 42 | - | - | -*** | - | - | - | - | - |
| 55 | - | - | -*** | - | - | - | - | - |
| 64 | - | - | -*** | - | - | - | - | - |

*Designation rated as follows, OD values of: <0.2 -; 0.21-0.4 +; 0.41-1.0 ++; 1.01-1.5 +++; and >1.501 ++++

**Serum tested at a dilution of 1:10

***These three sera tested as +, + and ++ respectively when tested at a dilution of 1:20.

The data presented in Table 5 suggest that an immunodominant HCV epitope is associated with DP-3. The known positive post-chimpanzee serum reacted strongly with DP-3 but failed to react with any of the other six peptides. All human sera which reacted strongly with DP-3 at a dilution of 1:100 also scored as positive at a dilution of 1:10 in the

commercial (Ortho) assay. Only one serum, Number 2, reacted weakly with DP-1, DP-4 and DP-5. In addition, three sera, which scored as negative in the Ortho assay, reacted with DP-3 at a dilution of 1:20.

In a second series of experiments a panel containing duplicate plasma sample obtained from a set of 19 patients defined serologically as NANBH-positive were tested. This set of 42 samples also contained two duplicate samples derived from two normal non-infected individuals. This panel was tested for antibody reactivity in wells coated with each individual synthetic peptide, DP-1 to DP6 and DP-8 to DP-18. The results are summarized in Table 6.

Table 6

RL Panel Screen on all Peptides

| | 1B | 2 | 3D | 4 | 5 | 6 | 8 | 9A | 9B | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RL 1 | | | + | | | | + | | + | + | | | | | | | | |
| RL 2 | | | | | | | | | | | | | | | | | | |
| RL 3 | | | | | | | | | | | | | | | | | | |
| RL 4 | | | | | | | | | | | | | | | | | | |
| RL 5 | | | + | | | | | | | | | | | | | | | |
| RL 6 | | | | | | | | | | | | | | | | | | |
| RL 7 | | | + | | | | + | +- | + | + | | | | | | | | |
| RL 8 | | | | | | | | | | | | | | | | | | |
| RL 9 | | | | | | | | | | | | | | | | | | |
| RL10 | | | + | | | | + | | | | | | | | | | | |
| RL11 | | | + | | | | | | | | | | | | | | | |
| RL12 | | | | | | | | | | | | | | | | | | |
| RL13 | | | + | | | | + | + | + | + | | | | | | | | |
| RL14 | | | | | | | | | | | | | | | | | | |

| | 1B | 2 | 3D | 4 | 5 | 6 | 8 | 9A | 9B | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RL15 | | | | | | | | | | | | | | | | | | |
| RL16 | + | | + | | | | + | | ++ | | | | | | | | | |
| RL17 | | | + | | | | + | | | | | | | | | | | |
| RL18 | | | + | | | | | | | | | | | | | | | |
| RL19 | | | + | | | | + | | | | | | | | | | + | |
| RL20 | | | + | | | | + | + | + | | | | | | | | | |
| RL21 | | | + | | | | | | | | | | | | | | | |
| RL22 | | | | | | | | | | | | | | | | | | |
| RL23 | | | + | | | | | + | + | + | | | | | | | | |
| RL24 | | | | | | | | | | | | | | | | | | |
| RL25 | | | | | | | | | | | | | | | | | | |
| RL26 | | | | | | | | | | | | | | | | | | |
| RL27 | | | + | | | | | | | | | | | | | | | |
| RL28 | | | + | | | | + | | + | | | | | | | | | |
| RL29 | | | + | | | | | + | + | + | | | | | | | | |
| RL30 | | | | | | | | | | | | | | | | | | |
| RL31 | | | | | | | | | | | | | | | | | | |
| RL32 | | | + | | | | + | +− | + | +− | | | | | | | | |
| RL33 | | | | | | | | | | | | | | | | | | |
| RL34 | | | | | | | | | | | | | | | | | | |
| RL35 | | | | | | | | | | | | | | | | | | |
| RL36 | | | | | | | | | | | | | | | | | | |
| RL37 | + | | + | | | | + | | + | + | | | | | | | | |
| RL38 | | | | | | | | | | | | | | | | | | |
| RL39 | | | + | | | | | | | | | | | | | | | |
| RL40 | | | | | | | | | | | | | | | | | | |
| RL41 | | | | | | | | | | | | | | | | | | |
| RL42 | | | + | | | | + | | | | | | | | | | + | |

Again it appears from the data that DP3, and in this case also DP-3D, contain a major immunodominant

HCV epitope. Further, a number of the sera tested also react with core associated peptides DP8, DP9A, DP9B, and DP10. One patient (duplicate RL 19 and RP42) responded to an NS5 epitope identified by peptide DP17.

The sensitivity of the ELISA assay, developed by coating wells with the DP-3 peptide, was analyzed by testing serial 10-fold dilutions of pre- and post-NANBH infection chimpanzee 174 sera. The results of this analysis are presented in Figure 16 (410 - m). A clear positive reaction was observed when post-chimpanzee 174 sera (post 174) was tested at a dilution of 1:1,000 with borderline activity noted at a dilution of 1:10,000. Pre-174 serum background gave only negligible absorbency reading. The post-174 serum was fractionated by gel chromatography on "SEPHADEX G-200." A significant level of activity was noted in the IgG containing fractions at a concentration of 1 μg/ml (Figure 16). Further, a low level of anti-HCV activity was also detected in the void volume from this fractionation: the void volume contained IgM fractions. Chimpanzee 174 (post) also reacted at a serum dilution of 1:100 with DP1 at an absorbance of 0.4.

The results of a titration curve of post chimpanzee 174 with wells coated with DP-1 is illustrated in Figure 17 (absorbency at 410). In Figure 17 the (+) designates post infection serum and the (X) designates pre-NANBH infection serum. Chimpanzee 174 serum is more than 10-fold more reactive with DP-3 as compared to reaction with DP-1.

A human plasma with a high level of reactivity for HCV was titrated using the DP-3 peptide ELISA assay described above. This plasma contained

positive antibody reactivity at dilutions of 1:10,000 and 1:100,000.

## EXAMPLE 11

## EPITOPE MAPPING OF AN IMMUNODOMINANT EPITOPE ASSOCIATED WITH PEPTIDE DP-3

As described above in Example 10, Tables 4 and 5, an immunodominant epitope was identified within the DP-3 peptide as a result of screening of a number of NANBH human sera and experimental HCV infected chimpanzee sera. Further, the antibody activity was not detected in this serum panel when tested with DP-2: DP-2 and DP-3 contain six overlapping amino acid residues, 1917-1922

Since no reactivity was observed with the DP-2 peptide, it was assumed that the six N-terminal residues of DP-3 were not associated with this epitope. In order to more precisely identify the DP-3 epitope reaction site, the following three overlapping peptides which encompass the 18 carboxy-terminal residues of DP-3 were synthesized: DP-3A, presented as SEQ ID NO:4; DP-3B, presented as SEQ ID NO:5, and DP-3C, presented as SEQ ID NO:6. Each of these three peptides were tested for direct binding with NANBH antibody positive sera and also for their ability to inhibit binding of anti-HCV antibody with the original DP-3 peptide.

Five human sera, RL1, RL7, RL13, RL19, and RL20, previously shown to react with DP-3, were tested (as described above) in wells coated with peptides D-3, DP-3A, DP-3B and DP-3C. The patterns of anti-peptide reactivity are illustrated in Figure 18 (absorbency at 410). The major DP-3 binding activity is associated with DP-3B.

Next a series of inhibition experiments were performed using the above peptides. Briefly, a final plasma dilution of 1:100, for each of the five plasma RL plasma listed above, was incubated with 20 μg/ml of each of the respective peptides. Subsequently, this mixture was tested for residual antibody reactivity in wells coated with DP-3. Figure 19 presents the data showing percent inhibition generated with the five plasma and 4 peptides. All 5 plasma were inhibited by 50% to 70% by DP-3. Two of the plasma were inhibited to similar levels with DP-3B and about 17% by DP-3C. On the other hand, none of these plasma were inhibited by Dp-3A.

Post chimpanzee 174 serum was also tested for binding and inhibition using the 4 peptides and the above protocols. The pattern of reactivity was similar to that seen with human serum RL20. The chimpanzee serum was further tested by performing the inhibition assay protocol using increasing amounts of each short peptide. The results of this assay are shown in Figure 20. The data show that increasing quantities of DP-3B (+) and DP-3C (*) both inhibit antibody binding with DP-3: DP-3A (closed rectangles) shows only low level inhibition. These results suggest two possibilities: (i) that antibody for both peptides DP-3B and PC-3C can be identified but that DP-3B binds antibody with higher avidity than DP-3C; or (ii) that two populations of antibodies may be present in this polyclonal chimpanzee anti-DP-3 serum, which recognizes overlapping Dp-3 epitopes.

EXAMPLE 12

SENSITIVITY AND SPECIFICITY OF THE DP-3 BASED ANTI-HCV ELISA ASSAY

Dr. Harvey Alter, at the Department of Transfusion Medicine clinical Center, National Institutes of Health, Bethesda, MD, assembled a double blind serum panel which contained a number of documented NANBH patient sera. This panel of 26 sera was obtained from Dr. Alter and screened using the DP-3 based anti-HCV ELISA assay. Briefly, the DP-3 peptide was fixed into wells of microtiter plates, the sera was diluted 1:100, added to the wells and incubated for 1 hr at 37°C. The sera were then withdrawn from the wells and the wells washed. Anti-human IgG labelled with HRPO was added to each well. The presence of bound antibody was detected by ABTS-30 min. develop read absorbency at 410 nm. The results of the above screening are summarized in Table 7.

Table 7

| Clinical Diagnosis | Concordance | Readings (1:100) |
|---|---|---|
| NANBH | 12/12 Positive ( 2) | 0.527 - 0.648 (10) over |
| Control | 14/14 Negative (14) | 0.026 - 0.102 |

(2) Negative by Original Chiron Antibody Assay
--No False Positives
--No False Negatives

As can be seen from the data presented in Table 7 a perfect concordance was observed when the double blind code was broken by Dr. Alter. All 12 sera

that were obtained from NANBH patients tested positive using the DP-3 based assay and the 14 additional sera derived from patients with a variety of ailments other than NANBH tested negative.

The two sera that tested with intermediate absorbency readings (0.527 - 0.648) had been obtained from a patient shortly after resolution of an acute infection. Therefore, Dr. Alter presumed that the patient was in an early stage of immune response.

## EXAMPLE 13

## ESTABLISHMENT OF OPTIMAL PARAMETERS FOR PEPTIDE-BASED ANTI-HCV ASSAY

The optimal concentration of the DP-3 peptide for microwell coating for use in ELISA assays was determined by coating wells using serial two-fold dilutions of DP-3 peptide in a series of different buffers. After coating, the wells were post-coated with a number of different standard agents useful for blocking non-specific protein binding, including normal goat serum (NGS), bovine serum albumin (BSA) or Blotto (50 g non-fat powdered milk in 1 L 0.05% (v/v) Tween-20/PBS).

The coated wells were tested against pre- and post- NANBH infection chimpanzee 174 sera diluted 1:100 and 1:1000. Optimal results were obtained with DP-3 diluted in bicarbonate buffer (10 mM $Na_2CO3-H_2O$, 30 mM $NaHCO_3$), pH 9.5, and post-coated with 10% NGS. Peak reactivity was determined based on the calculation of P/N ratios: the absorbency value of positive serum divided by absorbency value of negative serum tested at the same dilution. Peak reactivity was observed when the wells were coated with 500 ng of DP-3 peptide per well (Figure 21).

In order to further increase the sensitivity of the assay the effects of several other parameters on the assay where examined: for example, diluent, wash buffer, purity of the peptide, and concentration of the sera. Purification (de-salting) of the peptide by HPLC reduced the background level (C18 column, .1% TFA + 5% Acetic Nitrile, 10.1% TFA + 50% Acetic Nitrate in 45 minutes). A preferred well-wash buffer was found to be PBS containing 0.5% "TWEEN"-20, (Sigma). NGS and PBS were compared as diluents and PBS gave a lower background than the NGS. The above parameters of the assay can be easily modified to performed the assay at sera dilutions of 1:20 with low background using PBS/0.5% Triton X-100 (Sigma) as diluent.

## EXAMPLE 14

## SCREENING OF A KNOWN HCV DOUBLE-BLIND PANEL

A second sera panel consisting of 42 sera obtained from a number of documented NANBH patients and known normal sera were labelled in a double-blind fashion (designated the labelled RL Panel). The panel was tested at a 1:100 dilution of sera in the DP-3 ELISA and at a 1:10 dilution of sera when using the Ortho anti-HCV test kit.

The absorbance values for each individual serum are illustrated in Figure 22. Upon breaking the double-blind code the panel was seen to contain 21 serum samples, each present in duplicate. Nineteen pairs of the sera were derived from pedigreed NANBH patients and two pairs were obtained from normal individuals. The four normal sera were scored as negatives in the DP-3 assay. Thirty-two of the 38 NANBH sera (84.2%) were scored as being antibody

positive in the DP-3 assay using a serum dilution of 1:100 (Table 8).

The same serum panel was screened at a dilution of 1:10 using the Ortho anti-HCV assay. Using the Ortho assay, 26 of the 38 (65.4%) NANBH sera were scored as positive (Table 8). All Ortho assay positive sera scored as positive using the DP3 assay. The above data suggests that the DP-3 assay is more sensitive than the commercially available first generation Ortho anti-HCV assay.

Table 8

| Anti-HCV Serologic Assay* | Number Positive Number Treated | Percent Positive |
|---|---|---|
| Ortho Antibody | 26/38 | 65.4% |
| DP-3 Antibody | 32/38** | 84.2% |

*Plasma screened at a dilution of 1:10 in the Ortho assay and at a dilution of 1:100 in the DP3 assay.

**4 of these 32 reactive plasma samples were recorded as positive when tested at a dilution of 1:10 in the DP3 assay.

## EXAMPLE 15

## SCREENING OF A DOUBLE-BLIND PANEL CONTAINING BLOOD DONOR PLASMA HAVING DETERMINED ALT VALUES

A panel of blood donor plasma was assembled from a total of 82 units that had been rejected units on the basis of elevated ALT values. These plasma were screened for anti-HCV activity using 1) the first generation Ortho assay testing at a plasma

dilution of 1:10, and 2) the DP3 based assay testing at a plasma dilutions of 1:20 and 1:100.

The results of the assays were plotted as histograms. Histograms of the DP3 tested plasma at 1:100 dilution are illustrated in Figure 23 (OD = absorbency at 410 nM). Eight of 82 plasmas tested gave strong positive reactions. Another ten were scored as positive with OD readings ranging between 0.2 to 0.5. Each of the plasma which reacted at reduced levels were tested in two-fold dilutions with a starting dilution of 1:20. Each yielded a linear dilution curve as shown in Figures 24A and 24B (OD = absorbency at 410 nM). In addition, each low reactive plasma was inhibited by prior incubation with the soluble peptide DP3 (see Example 16 below).

Eighteen antibody positive plasmas were detected when the plasmas were tested at a dilution of 1:10. All plasma detected with the Ortho assay were strongly positive in wells coated with the DP3 peptide (Table 9).

Table 9

| Anti-HCV Assay | Number Positive Number Tested | Percent Positive |
|---|---|---|
| Ortho | 8/82* | 9.8% |
| DP-3 | 18/82 | 22.0% |

*All strongly positive in the DP-3 assay.

Accordingly, 9.8% and 22.0% of the panel were scored as anti-HCV positive as tested with the Ortho assay and the DP3 based assays, respectively.

## EXAMPLE 16

## DEVELOPMENT OF A CONFIRMATORY ASSAY USING COLD-PEPTIDE INHIBITION

The following protocol was developed to confirm that plasmas which reacted with the DP-3 based ELISA assay at low levels (0.2 - 0.5) are true positives.

Dilutions of plasma were pre-incubated with increasing quantities of DP3 peptide and then the plasma were tested in wells coated with DP3. For example, positive plasma were selected from the double blind RL panel and diluted 1:50. Equal volumes of buffer, or quantities of DP3 peptide to give a final concentration of 1 or 10 µg/ml were added to each respective plasma dilution and incubated for one hour at 37°C. These mixtures were then tested in DP3 coated wells: representative results are shown in Table 10.

Table 10

| Serum ID No. | Absorbency 1:100 | Percent Inhibition 1 µg | 1 µg |
|---|---|---|---|
| 10 | 1.215 | 83 | 95 |
| 12 | 0.476 | 42 | 70 |
| 17 | 1.239 | 73 | 95 |
| 33 | 0.140 | 44 | 51 |
| 36 | 0.145 | 47 | 57 |
| 38 | 0.439 | 57 | 70 |

Antibody reactivity was inhibited from 50 to 95% by prior incubation in the presence of uncoupled DP-3 peptide.

Next, both high and low reacting plasmas were pre-incubated, as above, at serum dilutions of 1:50 with an equal volume of DP-3 containing solution

consisting of increasing amounts of DP-3 peptide, resulting in a final plasma dilution of 1:100. The results from these assays are plotted in Figures 25A and 25B (O.D.). In these assays a high titer chimpanzee anti-HCV serum (post-174) along with the normal pre-bleed from the animal (pre-174) were included as controls. All plasmas derived from the High ALT Panel (447, 552 1.901, and 767 1.058; Example 15) were inhibited more than 50% by addition of 20 μg peptide (Figure 25A). In addition, low reacting plasmas 435 and 553 were also inhibited by more than 50%. However, the reactivity of plasma 850 was not significantly changed by prior incubation with the DP3 peptide (Figure 25B). Therefore, this plasma was recorded as a non-specific anti-HCV negative plasma with high background activity.

## EXAMPLE 17

## COMPARATIVE PERFORMANCE LEVELS OF THE DP-3 PEPTIDE BASED ASSAY WITH THE FIRST GENERATION ORTHO DISTRIBUTED ANTI-HCV ASSAY

Each of the plasmas contained in the RL double-blind panel (Example 14) were diluted in ten-fold dilutions, starting at a dilution of 1:10. The dilutions were then tested with the two anti-HCV assays, DP3-based and the first generation Ortho assay. The comparative results are shown in Table 11.

Table 11

| ORTHO | | | | DP-3 | | | |
|---|---|---|---|---|---|---|---|
| No. | Titer | No. | Titer | No. | Titer | No. | Titer |
| 1 | 100 | 28 | 1,000 | 1 | 10,000 | 28 | 10,000 |
| 2 | 10 | 6 | 10 | 2 | 100 | 6 | 100 |
| 3 | -- | 35 | NT | 3 | -- | 35 | NT |
| 4 | -- | 41 | -- | 4 | -- | 41 | -- |
| 5 | 100 | 39 | 100 | 5 | 10,000 | 39 | 10,000 |
| 7 | 1,00 | 32 | 1,000 | 7 | 1,000 | 32 | 1,000 |
| 8 | -- | 24 | -- | 8 | -- | 24 | -- |
| 9 | -- | 22 | -- | 9 | -- | 22 | -- |
| 10 | -- | 17 | -- | 10 | 1,000 | 17 | 1,000 |
| 11 | (10,000) | 27 | 1,000 | 11 | 1,000 | 27 | 1,000 |
| 12 | -- | 38 | -- | 12 | 100 | 38 | 100 |
| 13 | 100 | 20 | 1,000 | 13 | 10,000 | 20 | 10,000 |
| 14 | -- | 15 | -- | 14 | -- | 15 | -- |
| 16 | 100 | 37 | 100 | 16 | 1,000 | 37 | 1,000 |
| 18 | 1,000 | 21 | 1,000 | 18 | 1,000 | 21 | 1,000 |
| 19 | 1,000 | 42 | 100 | 19 | 100,000 | 42 | 10,000 |
| 23 | 100 | 29 | 1,000 | 23 | 1,000 | 29 | 1,000 |
| 25 | 10 | 34 | 10 | 25 | 1,000 | 34 | 100 |
| 26 | -- | 30 | -- | 26 | 10 | 30 | 10 |
| 31 | 10 | 40 | 10 | 31 | 100 | 40 | 100 |
| 33 | -- | 36 | -- | 33 | 10 | 36 | 10 |

As discussed above in Example 14, duplicates of each of 21 plasma were placed in this panel. Each assay had good reproducability between each of the duplicate samples tested. Based on this data, the DP3 based assay is in excess of ten-fold more sensitive than the Ortho assay for the detection of anti-HCV.

A second observation suggested by the comparative data shown in Table 11 is that the DP-3-

associated epitope elicits a highly specific antibody response: note paired patient plasmas 10/17 and 12/38. Reactivity was noted at dilutions of 1:1000 and 1:100, respectively, when tested with DP3, but no reactivity was noted with the Ortho assay.

## EXAMPLE 18

## COMPARISON OF THE RELATIVE SENSITIVITY AND SPECIFICITY OF THE DP3 AND THE ORTHO ANTI-HCV ASSAYS

A panel of donor blood plasmas was tested for anti-HCV at plasma dilutions of 1:10 by the Southwest Regional Blood Bank, San Antonio, Texas using the commercial first generation Ortho anti-HCV assay. The same panel of 362 plasma was tested at a plasma dilution of 1:100 using the DP3 based assay. The results of this test were sent to the Southwest Regional Blood Bank where all the results were compiled along with surrogate markers, such as, ALT, anti-HBc and HBsAg. These results are presented in Table 12.

All of the positives recorded with DP3 assay were tested with the peptide inhibition assay. All of the Ortho positive plasmas were tested with the commercial RIBA test (used to detect antibodies to recombinant proteins and fusion proteins, Ortho). All the plasma that gave a positive signal by either assay is shown in Table 12.

Table 12

Test Results*

of Blood Bank Panel

| ID# | DP-3 (1.4%)** | ORTHO (0.75%)** | ALT | ANTI-HBc | HBsAG |
|---|---|---|---|---|---|
| 435 | + | - | - | - | - |
| 447 | + | - | + | - | - |
| 552 | + | + | - | - | - |
| 746 | - | +*** | - | - | - |
| 750 | + (NS) | - | - | - | - |
| 767 | + | + | - | - | - |
| 553 | + | - | - | + | - |

*Blood Donor Panel - 362 sera.

**Total percent positive in the panel of 362 sera.

***Shown to be negative when tested with the Recombinant Immune Blot Assay (RIBA) recently developed by Chiron.

Of the six plasmas recorded as anti-HCV positive in the DP3 assay, five were confirmed by specific antigen inhibition. One DP3 reactive plasma (750) was shown to a non-specific positive by DP3 inhibition and also did not react in the Ortho assay. Three positives were noted when tested by Ortho, one of which was scored as non-specific because it did not react in a positive pattern with the RIBA confirmatory strip. A summary of the results is presented in Table 13.

Table 13

| Plasma ID No. | DP3 OD | Reactivity Score* | Ortho OD. | Reactivity Score** |
|---|---|---|---|---|
| 435 | 0.462 | Conf. Pos. | 0.012 | Negative |
| 447 | >2.0 | Conf. Pos. | 0.015 | Negative |
| 552 | 1.901 | Conf. Pos. | 0.974 | Conf. Pos. |
| 553 | 0.238 | Conf. Pos. | 0.005 | Negative |
| 746 | 0.052 | Negative | | Non-Specific |
| 750 | 0.345 | Non-Specific | 0.017 | Negative |
| 767 | 1.058 | Conf. Pos. | 1.545 | Conf. Pos. |

*Score = original reactivity scored as conf. pos. or non-specific by peptide inhibition.

**Score = original reactivity scored as conf. pos. or non-specific by testing with a Recombinant Immune Blot Assay (RIBA) developed by Chiron.

A positive rate of 1.4% was observed with the DP3 assay whereas 0.6% of the panel was recorded as antibody positive in the Ortho test. Both assays recorded one non-specific positive (Table 14).

Table 14

| 5 Confirmed Positive with DP3 Assay (1.4%)<br>2 Confirmed Positive with ORTHO Assay (0.6%) |
|---|
| DP3 Positive; ORTHO Positive = 2 samples |
| DP3 Positive; ORTHO Negative = 3 samples |
| DP3 Positive; ORTHO Negative = 1 (DP-3 non-specific) |
| DP3 Negative; ORTHO Positive = 1 (ORTHO non-specific) |

## EXAMPLE 19

## IDENTIFICATION OF HCV ENVELOPE AMINO ACID SEQUENCES THAT REACT WITH CONVALESCENT SERA

As discussed in Example 1, the majority of HCV infected chimpanzees and humans (Burk et al. 1984A) develop a long lasting chronic infectious stage. Many of these chronic infections are life-long. This is substantiated by the fact the report of Burk et al (1984B) that asymptomatic chimpanzees can be reinfected by challenge with acute stage homologous sera challenge. This result indicates that most HCV infected hosts (man or chimpanzee) fail to produce a protective antibody. Researchers who have studied the specificity of protective (neutralizing) antibody members of the Flavivirus or Pestivirus families have reported neutralizing antibody produced to the envelope viral glycoproteins (reviewed in Fields et al. 1990).

Experiments performed in support of the present invention showed that antibody was not detected to any of the six envelope glycoproteins tested against NANBH-implicated plasma (Example 10, Table 6): although more than half produced antibody to HCV core or non-structural proteins.

Based on previous observations (Burk et al, 1984B) three human patients (A1115, Mayfield and Hart) appeared to have developed convalescence based on 1) their clinical history and 2) the fact that they produced antibody after a prolonged period of chronicity, which reacted with a cytoplasmic antigen within acute stage HCV infected chimpanzee hepatocytes. Further, one plasma (ALT 2) was identified in the high ALT plasma panel (Example 15) which reacted in a preliminary screening against DP6, an E1 associated peptide.

The above plasmas, A1115, Hayfield, Hart and ALT2, were screened at a 1:20 dilution against the six synthetic peptides associated with HCV E1 (DP-12, DP-13, DP-16) and E2 (DP-14, DP-15 and DP-6). A summary of the reactivity of these peptides towards the above plasmas is presented in Table 16.

Table 16

| Envelope Protein | Peptide Designation | Amino Acid Residue Nos. | Convalescent Human Antibody Reactivity |
|---|---|---|---|
| e1 | DP-12 | 233-251 | +- |
| e1 | DP-13 | 291-317 | +- |
| e1 | DP-16 | 185-210 | ++ |
| e2 | DP-14 | 439-466 | +- |
| e2 | DP-15 | 467-493 | + |
| e2 | DP-16 | 641-660 | + |

Peptide DP-16 (E1) and peptides DP-15 and DP-6 (E2) reacted with each of the four sera. Three of these sera had the properties consistent with a convalescent stage of HCV disease based on the observations summarized in Figure 1. Each of these four plasmas, in addition to an HCV antibody negative plasma derived from the High ALT panel and a normal human plasma, were titrated in two-fold dilutions to the three reactive peptides, DP-6, DP-15 and DP-16. The results of these titration experiments are illustrated in Figures 26, 27 and 28. In general, each of the four plasma have an end point titer of approximately 1:160.

If these four plasmas are truly convalescent and, therefore, contain neutralizing antibody, it follows that one or more of these three peptides contain an associated neutralizing epitope which will be useful in vaccine production.

## EXAMPLE 20

## DEVELOPMENT OF AN IMMUNOASSAY FOR DETECTION OF HCV NS4 ANTIGEN

This example describes the use of an HCV non-structural protein antigen to design an antigen-based detection system for HCV.

The basic design of the immunoassay is illustrated in Figure 30. Microwells were coated with IgG derived by gel filtration from high titer rabbit anti-DP3D sera. Typically, sera are initially fractionated by addition of ammonium sulfate. The supernatant is then passed over a gel filtration column (Pierce) and the IgG containing fraction identified by absorbance ($A_{280}$). IgG molecules can be isolated by a number of standard procedures (Garvey, *et al.*), including affinity chromatography, or by the use of commercially available kits (Pierce). The wells of microtiter plates (Dynatech) were coated with the rabbit IgG antibody as follows (Harlow, *et al.*). Approximately 50 µl of purified rabbit IgG (approximately 20 µg/ml), in phosphate buffered saline (PBS) (Maniatis, *et al.*) is added to each well of polyvinylchloride plates. The plates are sealed and incubated for either 4 hours at room temperature or overnight at 4°C. Alternatively, polystyrene 96 well plates "IMMULON II" (PGC) (or suitable plastic plates, Corning Biotechnology, Corning NY) were coated with 5 ug/mL (100 µL per well) rabbit IgG in 0.1 M carb/bicarbonate buffer, pH 9.5, the plates sealed with parafilm and stored at 4°C overnight. The wells can also be coated with an anti-rabbit IgG antibody, followed by addition of the rabbit IgG.

After incubation the wells are washed twice with binding buffer (PBS or carb/bicarbonate

buffer). To each well is added approximately 200 µl of 3% bovine serum albumin (BSA) in PBS, containing 0.02% sodium azide. The plates were then incubated for approximately 2 hours at room temperature and the liquid removed.

The antibody coated wells were then incubated with the test samples, such as, DP3D peptide, HCV infected tissue culture media, human sera, or chimpanzee sera, for 1 hr. After incubation, DP3D-HRPO (horse radish peroxidase) conjugate was added to each well.

Peptide-HRPO conjugates were formed using commercially available activated HRPO (Pierce). Alternatively, HRPO is coupled to peptides using the techniques discussed in Example 8: (i) the glutaraldehyde technique that links through epsilon-amino acid groups on lysine residues, or other free amino groups (Pierce); or (ii) a two-step procedure using m-maleimidobenzoyl sulfosuccinimide ester (sulfo-MBS) to link through free disulfide linkages (Pierce). As also noted above, for peptides that lacked any free epsilon-amino groups or sulfhydryl groups, a cysteine residue or a lysine residue was added to the N-terminus of the peptide.

Combination of the conjugate to the solid phase antibody coat was detected by the addition of the substrate-2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Pierce), essentially as described in Materials and Methods above. The presence of a DP3D associated viral antigen was identified by color diminution due to successful inhibition of binding DP3D-HRPO.

A. DP3D.

The above protocol was first tested by addition of increasing quantities of free DP3D peptide. As shown in Figure 31, the addition of the DP3D peptide at a concentration of 10 μg/ml inhibited reactivity with DP3-HRPO by 68%. A linear inhibition curve was observed over the range of 10 μg/ml to 0.001 μg/ml. One ng/ml DP3D inhibited the reaction by 8%.

B. Epitope Mapping of DP3 Defined Epitope.

A number of overlapping peptides have been made and tested for their reactivity with anti-HCV antibody and three, DP3D, DP3E and DP3F have been tested for their utility as probes in the inhibition antigen assay. The results are summarized in Table 17.

Table 17

| Peptide | Sequence* | Reactivity for Detection of Anti-HCV | NS4 Ag |
|---|---|---|---|
| DP2 | 1902-1926 | - | ND |
| DP3 | 1917-1940 | ++++ | +++ |
| DP3A | 1922-1931 | - | ND |
| DP3B | 1928-1935 | +++ | ND |
| DP3C | 1933-1940 | + | ND |
| DP3D | 1922-1945 | ++++ | ++++ |
| DP3E | 1928-1951 | ++ | +++ |
| DP3F | 1932-1955 | - | -** |

*Carboxy terminus of C-100 is 1931 (Proline)
**Peptide insoluble at neutral pH.

EXAMPLE 21

ANTIGEN CONFIRMATORY ASSAY

A confirmatory assay was designed and tested to confirm positive antigen reactive plasma. The basic design is a blocking of the DP3-HRPO inhibition assay (Blocking of Inhibition) by addition of anti-DP3D positive sera to antigen positive plasma before

testing in the antibody coated plates. Feasibility of this design was demonstrated by prior addition of 1) rabbit anti-DP3D, and 2) antibody positive plasma, to different concentrations of synthetic peptides. Feasibility for this protocol was observed with both antisera.

The reagents for the confirmatory assay were evaluated by titration. Two HCV antibody positive sera were tested for their ability to block the inhibition reaction. The sera were human ALT 2 and X174. At a dilution of 1:1280 the chimpanzee ALT 2 serum blocked the inhibition of 100 ng of DP3D by 83% as shown in Figure 35B. The X174 serum was only titered to 1:80, due to the limitation of space in the assay. At 1:80 there was 99% blocking.

## EXAMPLE 22

## DETECTION OF HCV NS4 ANTIGEN IN PLASMA DERIVED FROM EXPERIMENTALLY HCV INFECTED CHIMPANZEES

Plasma derived from six chimpanzees during the period when they demonstrated an elevation in their ALT values, that is, the acute phase, was used in the assay described in Example 20 to examine the effectiveness of the assay to detect the HCV antigen during the acute phase. The six chimpanzees were infected as follows. Chimpanzees 194 and X7 were each inoculated with $10^{2.5}$ chimpanzee infectious doses of human infectious inoculum, Hutchinson. Chimpanzee 174 was inoculated with 10 ml acute phase plasma derived from chimpanzee X7. Chimpanzee 268 was inoculated with 2 ml acute phase chimp 174 plasma. Chimpanzee 198 was inoculated with 50 ml of chimp 268 acute phase plasma. Chimpanzee 196 was inoculated with the following materials; day 0 and 84 with a HCV infected TC media pool concentrated by

ultrafiltration and on day 168 with 50 ml chimp x174 acute phase plasma. Chimpanzee 623 was injected with 1 ml of sucrose gradient purified TC propagated virus on days 0 and 104. This was followed by inoculation with 100 ml homologous acute phase plasma on day 177 with 50 ml of acute phase plasma derived from chimpanzee X7 on day 321.

Plasma from four of the six animals inhibited the DP3D-HRPO reaction (Example 20). Using the DP3D inhibition curve as a standard, the level of DP3 defined antigen ranged from 18-46 ng/ml (Figure 32).

A number of HCV parameters were evaluated for sequential plasma derived from chimpanzee 196, who was experimentally infected with tissue culture propagated HCV. These parameters are summarized in Figure 33: open blocks - histogram, ALT u/ml (Hollinger, 1984); (+), HCV DP3D antigen, percent inhibition (Example 20); open blocks - plotted line, HCV Ab levels as determined by DP3D based ELISA and PCR (Mullis; Mullis, *et al.*) (+/-/nd, nd = not determined), detection of HCV nucleic acid using polymerase chain reaction (Weiner et al. 1990; Garson et al. 1990).

To further illustrate the utility of the antigen-based detection assay, sequential plasmas derived from two additional animals (623 and 174) were also tested for ALT values, DP3D antigen and anti-DP3D. These profiles are shown in Figures 34 and 35. Figure 34, Chimpanzee 623, shows the results of antibody tests on the sequential sera, the ALT values, and the HCV Ag percent inhibition: these values were all determined as described above. Figure 35 shows a similar analysis of sequential obtained sera for Chimpanzee 174.

EXAMPLE 23

DETECTION OF HCV ANTIGEN IN HUMAN PLASMA

A panel containing 82 human plasma samples derived from individuals with elevated ALT values and a second panel containing 228 plasmas from normal blood donors were screened for the presence of HCV antigen (Example 20). The results of the screens are presented in Table 18.

Table 18

| HIGH ALT PANEL | | |
|---|---|---|
| Total of 82 Samples<br>6 of 82 samples are positive for inhibition | | |
| ID | % Inhibition | Equivalent Peptide Inhibition nG/mL |
| ALT 5 | 37 | 10 nG/mL |
| ALT 64 | 37 | 10 nG/mL |
| ALT 38 | 15 | 1 nG/mL |
| ALT 4 | 14 | 1 nG/mL |
| ALT 10 | 13 | 1 nG/mL |
| ALT 17 | 13 | 1 nG/mL |
| BLOOD DONOR PANEL | | |
| 221 Samples<br>2 of 221 samples tested are positive for inhibition | | |
| ID | % Inhibition | Equivalent Peptide Inhibition nG/mL |
| 344 | 38 | 10 nG/mL |
| 730 | 39 | 10 nG/mL |

All the samples in Table 18 that tested positive for inhibition using the HCV antigen detection assay were also tested for their reactivity using (i) a DP3-based antibody capture assay and (ii) the commercially available Ortho

anti-HCV assay which utilizes the C-100 protein. The C-100 protein is a fusion protein which was constructed from coding sequences derived from 3 overlapping HCV clones and coding sequences for human super oxide dismutase (SOD) (Houghton et al. 1989). C-100 contains amino acids 1569-1931 relative to the HCV polyprotein sequence reported in Houghton et al. (1990). A HCV solid phase radioimmunoassay (RIA) was developed with the recombinant protein and was used to test a panel of human sera (Kuo et al., 1989). All samples positive for inhibition using the DP3 antigen detection assay were negative for antibody by both Chiron and DP3-antibody capture assays.

To ascertain the specificity of these eight plasma samples each was tested in the blocking confirmatory assay described above. The confirmatory assay was performed by prior incubation of the positive reactant plasma with a 1:500 final dilution of an anti-HCV positive human plasma. The mixture was then tested in the antigen assay and a positive was confirmed by blocking of the ability of the sample to inhibit the DP3D anti-DP3D reaction. The results are shown in Table 19.

Table 19

High ALT Panel/Donor Panel
Screened by Antigen Assay

| Sera | Inhibition | Peptide Equivalent nG/mL | Confirmed |
|---|---|---|---|
| ALT1 | - | 0 nG | - |
| ALT4 | ± | 1 nG | + |
| ALT5 | + | 10 nG | + |
| ALT10 | ± | 1 nG | - |
| ALT17 | ± | 1 nG | - |
| ALT38 | ± | 1 nG | - |
| ALT64 | + | 10 nG | + |
| Donor # | | | |
| 942 | - | 0 nG | - |
| 35 | - | 0 nG | - |
| 344 | + | 10 nG | + |
| 730 | + | 10 nG | + |

For each sample a standard curve for antigen concentration (Example 20) and controls were performed.

EXAMPLE 24

DETECTION OF HCV NS4 ANTIGEN IN TISSUE CULTURE (TC) MEDIA DERIVED FROM HEPATOCYTES INFECTED WITH HCV

To increase the sensitivity of this assay, wells were coated with rabbit anti-DP3D purified by affinity chromatography utilizing "SEPHAROSE 4B" (Pharmacia) conjugated to DP3D (as per manufacturer's instructions). The standard curve is shown in Figure 35C. It is noted that the reaction of DP3D-HRPO conjugate with affinity purified anti-DP3D coated wells can be inhibited 100% by

incubation of 100 μg/ml DP3D. The end sensitivity of this assay using DP3D is 100 pg/ml.

A defined serum-free media (DSFM), which facilitated the *in vitro* long-term cultivation of normal marmoset, cynomolgus, baboon, chimpanzee, and human hepatocytes, has been previously described (Lanford et al. 1989, herein incorporated by reference; Patent Application Serial No. 07/222,569, filed 21 July 1988, herein incorporated by reference; and co-pending, co-owned US Patent Application Serial No. 07/504,171, filed 3 April 1990, herein incorporated by reference; Jacob et al. (1990, 1991)).

Briefly, the *in vitro* cell culture sustains primate hepatocytes in a serum-free medium comprising a basal cell culture medium (such as William's media E, Gibco BRL), a hepatocyte proliferogen (such as liver growth factor, Collaborative Research), serum albumin, a corticosteroid (such as hydrocortisone), one or both of somatotropin or prolactin, a growth/releasing factor, cholera toxin and ethanolamine. The source of the infectious NANBH inoculum was the Hutchinson strain (Feinstone et al. 1981). The presence of HCV in test cell supernatant fluids was supported as follows: 1) by infection of a normal chimpanzee using the test cell supernatant fluids; 2) by observation of enveloped 39-60nm virion particles; and 3) by sequencing of cloned viral RNA. Infected chimpanzee hepatocytes were isolated and grown in DSFM using our standard methods (Jacob, et al., 1990 and 1991).

A pool of media (designated TC in Table 19) derived from days 3-27 of tissue culture supernatants harvested at two day intervals from HCV

infected hepatocytes was tested for HCV DP3D associated NS4 antigen. Media derived from HBV infected hepatocytes was used as a negative control (Jacob *et al.*, 1989). The results of HCV antigen detection assay are presented in Table 20.

Table 20

| Virus Source | PCR | Average O.D. | % Inhibition |
|---|---|---|---|
| HBV, T.C. | - | 0.331 | - |
| HCV, T.C. unpurified | + | 0.152 | 54 |
| HCV, T.C. purified | + | 0.25 | 25 |

The HCV virus present in the media was purified by recover from the interface between a 20% and a 68% sucrose layer (Jacob *et al.*, 1991). Approximately 64% of the soluble antigen was lost during this purification step (Table 20).

The temporal synthesis of HCV NS4 antigen was investigated by testing sequential TC media derived at two day intervals from *in vivo* infected hepatocytes derived from a chronic HCV infected chimpanzee (animal No. 198). The results are shown in Figure 36. One control was the use of media from un-infected hepatocyte cells; 18 samples were used to determine the baseline of the assay, i.e., 0% inhibition. The values presented in Figure 36 are the average of triplicate values.

Sequential TC medias, derived from HCV infected hepatocytes from chronic chimpanzee 2246, were screened for DP3D associated HCV antigen. The results are illustrated in Figure 37. As noted previously in media from hepatocytes derived from a chronically HCV infected chimp, two cycles of

antigen production were noted, day 1 through 5, and days 5 through 15.

## EXAMPLE 25

## UTILIZATION OF ANTIGEN ASSAY FOR DRUG SCREENING FOR HCV

The HCV antigen detection assay of the present invention was used to assay the effects of drugs on the production of viral antigen *in vitro*.

Cultured hepatocytes derived from a chronic HCV infected chimpanzee (animal No. 2246) were treated with a number of drugs on day 3, including ribavirin and interferon (Table 20). The cells were maintained on these drugs thereafter, and media, harvested on days 5 and 12, were tested for HCV antigen (Example 20). The results of the antigen detection assay are presented in Table 21.

Table 21

HCV Ag Production in Absence or Presence of Drugs

| Media | Antigen nG/mL Day 5 | Day 12 |
|---|---|---|
| Serum Free Media | 0.3 | 1.2 |
| Ribavarin 1× | 0.15 | 0.45 |
| Ribavarin 5× | 0.15 | 0.45 |
| Interferon 1× | 0.9 | 0 |
| Interferon 1x | 0 | 0 |

The temporal synthesis of HCV NS4 antigen was investigated by testing sequential pooled media derived at two day intervals from *in vivo* infected hepatocytes derived from a chronic HCV infected chimpanzee (animal No. 2246). The results are shown

in Figure 37. As above, one control was the use of media from un-infected hepatocyte cells; 18 samples were used to determine the baseline of the assay, i.e., 0% inhibition. The values presented in Figure 37 are the average of triplicate values.

## EXAMPLE 26

## DEVELOPMENT OF AN IMMUNOASSAY FOR HCV STRUCTURAL (CAPSID) ANTIGEN

The peptides presented in Table 22 have been tested in the antigen inhibition assay, essentially as described in Example 20A, for use in an antigen detection assay targeted for the HCV capsid protein.

Table 22

| Peptide Designation | Amino Acid Residue No. | Sequence Listing No. | Putative HCV Domain |
|---|---|---|---|
| DP9A | 35-55 | 13 | C |
| DP9B | 51-75 | 14 | C |
| DP10 | 98-121 | 15 | C |
| DP11 | 146-169 | 16 | C |

Rabbit antisera was prepared to each respective peptide in Table 22. The resulting antibodies were affinity purified on "SEPHAROSE 4B" matrixes (Pharmacia, Piscataway NJ) conjugated to each respective peptide. Wells were coated with affinity purified rabbit anti-DP11 (Example 8). The probe reagents were generated by conjugation of each respective peptide to horseradish peroxidase (HRPO) using a heterobifunctional cross-linker (SPDP), as described above (Example 20).

The assay design was similar to that described above for the NS4 specific antigen detection test (Example 20).

Sequential plasma samples derived from an HCV infected chimpanzee (No. 196) were tested for presence of detectable capsid antigen as described above for the DP3 antigen (Example 20). The results of these inhibition assays are shown in Table 23.

Table 23

| Test Sample | DP9A | | DP9B | | DP10 | | DP11 | |
|---|---|---|---|---|---|---|---|---|
| | OD | %INH | OD | %INH | OD | %INH | OD | %INH |
| 10 | 0.000 | 100 | 0.000 | 100 | 0.000 | 100 | 0.000 | 100 |
| 1 | 0.000 | 100 | 0.053 | 87 | 0.030 | 77 | 0.014 | 93 |
| 0.1 | 0.089 | 70 | 0.102 | 75 | 0.041 | 68 | 0.029 | 85 |
| 0.01 | 0.216 | 26 | 0.223 | 45 | 0.081 | 37 | 0.043 | 78 |
| 0.001 | 0.235 | 20 | 0.286 | 30 | 0.083 | 35 | 0.086 | 56 |
| 0.0001 | 0.253 | 14 | 0.323 | 21 | 0.104 | 19 | 0.124 | 36 |
| 0 | 0.293 | 0 | 0.408 | 0 | 0.128 | 0 | 0.194 | 0 |
| *06/20/88 | 0.444 | 0 | 0.470 | 0 | 0.299 | 0 | 0.220 | 0 |
| 07/18/88 | 0.440 | 1 | 0.453 | 4 | 0.297 | 1 | 0.222 | 0 |
| 08/15/88 | 0.465 | 0 | 0.477 | 0 | 0.326 | 0 | 0.226 | 0 |
| **10/17/88 | 0.472 | 0 | 0.506 | 0 | 0.338 | 0 | 0.127 | 42 |
| 11/15/88 | 0.301 | 32 | 0.366 | 22 | 0.170 | 43 | 0.080 | 64 |
| 12/12/88 | 0.526 | 0 | 0.538 | 0 | 0.335 | 0 | 0.121 | 45 |
| 04/03/89 | 0.456 | 0 | 0.463 | 1 | 0.343 | 0 | 0.107 | 52 |
| 08/07/89 | 0.417 | 6 | 0.444 | 5 | 0.361 | 0 | 0.126 | 43 |
| 09/18/89 | 0.393 | 11 | 0.443 | 6 | 0.160 | 47 | 0.105 | 52 |
| 03/12/90 | 0.451 | 0 | 0.473 | 0 | 0.268 | 10 | 0.194 | 12 |
| 05/21/90 | 0.468 | 0 | 0.538 | 0 | 0.325 | 0 | 0.228 | 0 |
| 06/18/90 | 0.429 | 3 | 0.492 | 0 | 0.305 | 0 | 0.137 | 38 |

*Pre-bleed sample.

**First significant ALT elevation noted on 10/10/88.

It is of note that antigen was detected with each respective peptide assay following the first significant elevation in ALT values (11/15/88) and at one later date (09/18/89). However, the most striking observations were noted with peptide DP11 where capsid antigen was detected initially on day 11/16/88, and throughout the test period (in excess of 700 days) with one exception (05/21/90).

## EXAMPLE 27

## EXCLUSION OF ANTI-CAPSID ANTIBODY INTERFERENCE FOR DETECTION OF CAPSID ANTIGEN

A panel of plasmas derived from blood donors, excluded due to elevated ALT values (82 samples), were screened against all of the synthetic peptides presented in Table 3, including DP3 but not DP3A through DP3F. Among the capsid associated synthetic peptides (Table 3, "C"), the following antibody activity was noted: DP8, 7/82; DP9A, 5/82; DP9B, 5/82; and DP10, 2/82. None of the eight individual plasma which contained detectable antibodies to one or more of these peptides reacted to DP11.

This result suggests that the epitope associated with DP11 (HCV, amino acid residues 146-169) is not immunogenic in humans infected with HCV. However, rabbits immunized with immunogen prepared by conjugating DP11 to keyhole limpet hemocyanin, produce a vigorous immune response to DP11. Based on the above observations, DP11 was pursued as a candidate for the development of a HCV capsid specific antigen immunoassay.

EXAMPLE 28

INHIBITION HCV ANTIGEN ASSAY PROTOCOL USING DP11

Microtiter plates ("IMMUNOLON II," Dynatech Laboratories, Inc.) were coated with affinity purified rabbit anti-DP11 at a protein concentration of 0.4 μg/ml, 50 μl per well. Rabbit anti-DP11 was purified by affinity chromatography using DP11 coupled to Sepharose by sodium cyanoborohydride (Pharmacia). The plates were covered and incubated overnight at 4°C.

The wells were then emptied by aspiration and blocked with 10% normal goat serum in 0.01 M phosphate buffered saline pH 7.2 (PBS), 350 μl per well. The plates were incubated at 37°C for 1 hour then washed five times with PBS/Tween-20 (PBS containing 0.5% Tween-20).

Samples were diluted 1:2 with PBS/Tween-20. Fifty μl per well of positive control standards were added; unknown samples were performed in triplicate. Synthetic peptide DP11 was purified by HPLC (Multiple Peptide Systems). A standard curve was generated from 10-fold dilutions of a stock 1.0 μg/ml solution of DP11 in neat, heat-inactivated, normal human serum plus 0.01% thimerosal: positive standards contained 0.001 to 1.0 μg/ml DP11. The negative control was heat inactivated serum + 0.01% thimerosal.

The plate was covered and incubated at 37°C for 1 hour then washed five times with PBS/Tween-20. To each well 100 μl of DP11/HRPO probe diluted 1:500 in PBS was added. The DP11/HRPO probe was DP11 conjugated to HRPO with SPDP (Pierce). The reaction was incubated at room temperature for 30 minutes. The plate was washed five times with PBS/Tween-20.

To each well 100 μl of ABTS was added. The plate was incubated for 30 minutes at room temperature. The reaction was stopped after 30 minutes with 5% lauryl sulfate sodium, salt in distilled water. The reactions were then quantitated by measuring the optical density for each well at 410 nm using a reference filter of 490 nm. Results were calculated using following formula:

$$1-\frac{\text{(Corrected* Exper Mean OD)}}{\text{(Corrected Blank Mean OD)}}\times 100$$

*Correction = subtraction of mean OD of 1 ng positive control.

## EXAMPLE 29

## ANALYSIS OF HCV INFECTED HUMAN SERA USING THE DP11-BASED INHIBITION ASSAY

Three HCV seroconversion panels (4811, 4812, and 4813), each derived from a single human donor who was inadvertently infected with HCV infected human RBC preparations (supplied by Serologicals, Clarkston, GE), were analyzed for capsid antigen using the above-described DP11 based inhibition assay.

The first serum sample was used as the negative control for each respective panel. The first sample is indicated as day 0 in Figures 38, 39 and 40. Dr. Richard Newhouse (Serologicals) confirmed that vial 1 in each panel contained plasma obtained before inoculation with the HCV contaminated red blood cells (*i.e.*, they represent pre-bleed samples).

The results of the DP11 inhibition values are presented in Figures 38 (donor 4811), 39 (donor 4812) and 40 (donor 4813). The inhibition data is

plotted along with ALT and anti-HCV data (Ortho Kit). As can be seen from the results, in all three individuals low levels of DP11 associated antigen was detected in sample 2. In two panels the signal disappeared (Figures 38, days 6 and 10; Figure 40, days 13 and 16) and then became positive again (Figure 38, day 13; Figure 40, day 23). Individual No. 4812 (Figure 39) remained positive from day 4 through day 34.

At the time corresponding roughly with the elevation of the ALT values in each person (Figure 38, day 46; Figure 39, day 41; and Figure 40, day 34), antigen became undetectable. Upon resolution of the pathology, as noted by decline of the ALT values, all three individuals again developed positive antigen reactivity which was noted through the last sample provided for each individual (Figure 38, day 363; Figure 39, day 321, and Figure 40, day 397).

## EXAMPLE 30

## DETECTION OF NS4 AND CAPSID IN A SINGLE COMBINED IMMUNOASSAY

An immunoassay was developed for the simultaneous detection of both NS4 and capsid antigen. A 1:1 antibody mixture of rabbit anti-NS4 and anti-capsid was used as probe. The sensitivity of this assay was determined by testing a dilution series of a mixture of DP3D-DP9B peptides. The antigen-reporter complex was a mixture of DP3D-HRPO and DP9B-HRPO (both described above). The standard curve, shown in Figure 41, indicates that this assay has a sensitivity of less than 100 pg/ml. It should be noted that the peptide mixture was diluted in neat normal human plasma.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP1, amino acids 1694 to 1717 of HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Ile Pro Asp Arg Glu Val Leu Trp Arg Glu Phe Asp Glu Met Glu
1 5 10 15

Glu Cys Ser Gln His Leu Pro Trp
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP2, amino acids 1902 to 1922 of HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Arg Arg His Val Gly Pro Gly Glu Gly Ala Val Gln Trp Met Asn
1 5 10 15

Arg Leu Ile Ala Phe
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus

(C) INDIVIDUAL ISOLATE: DP3, amino acids 1917 to 1940 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Arg Leu Ile Ala Phe Ala Ser Arg Gly Asn His Val Ser Pro Thr
1 5 10 15

His Tyr Val Pro Glu Ser Asp Ala
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3A, amino acids 1922 to 1937 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ser Arg Gly Asn His Val Ser Pro
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3B, amino acids 1928 to 1935 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn His Val Ser Pro Thr His Tyr Val
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3C, amino acids 1933 to 1940 of

the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His Tyr Val Pro Glu Ser Asp Ala
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3D, amino acids 1922 to 1946 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Ala Ser Arg Gly Asn His Val Ser Pro Thr His Tyr Val Pro Glu
1 5 10 15

Ser Asp Ala Ala Ala Arg Val Thr
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP4, amino acids 1476 to 1493 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Thr Leu Pro Gln Asp Ala Val Ser Arg Thr Gln Arg Arg Gly Arg Thr
1 5 10 15

Gly Arg

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP5, amino acids 1488 to 1506 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Gly Arg Thr Gly Arg Gly Lys Pro Gly Ile Tyr Arg Phe Val Ala
1 5 10 15

Pro Gly Glu

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP6, amino acids 641-660 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Ala Ala Cys Asn Trp Thr Arg Gly Glu Arg Cys Asp Leu Glu Asp
1 5 10 15

Arg Asp Arg Ser
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Flavivirus
(C) INDIVIDUAL ISOLATE: DP7, conserved Flavivirus sequence, HCV unrelated

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Cys Lys Pro Pro Phe Gly Asp Ser Tyr Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP8, amino acids 1-21 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn
1 5 10 15

Arg Arg Pro Gln Asp
20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP9A, amino acids 35-55 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala Thr Arg
1 5 10 15

Lys Thr Ser Glu Arg
20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP9B, amino acids 51-75 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro Ile
1 5 10 15

Pro Lys Ala Arg Arg Pro Glu Gly Arg Thr
20 25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP10, amino acids 98-121 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Thr Asp Pro Arg
1 5 10 15

Arg Arg Ser Arg Asn Leu Gly Lys
20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP11, amino acids 146 to 169 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly Ala Ala Arg Ala Leu Ala His Gly Val Arg Val Leu Glu Asp Gly
1 5 10 15

Val Asn Tyr Ala Thr Gly Asn Leu
20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP12, amino acids 233 to 251 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Asn Ala Ser Arg Cys Trp Val Ala Met Thr Pro Thr Val Ala Thr
1 5 10 15

Arg Asp Gly

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP13, amino acids 291 to 317 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Phe Thr Phe Ser Pro Arg Arg His Trp Thr Thr Gln Gly Cys Asn Cys
1 5 10 15

Ser Ile Tyr Pro Gly His Ile Thr Gly His Arg
20 25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP14, amino acids 439 to 466 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala Gly Leu Phe Tyr His His Lys Phe Asn Ser Ser Gly Cys Pro Glu
1 5 10 15

Arg Leu Ala Ser Cys Arg Pro Leu Thr Asp Phe Asp
20 25

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP15, amino acids 467 to 493 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gln Gly Trp Gly Pro Ile Ser Tyr Ala Asn Gly Ser Gly Pro Asp Gln
1 5 10 15

Arg Pro Tyr Cys Trp His Tyr Pro Pro Lys Pro
20 25

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP16, amino acids 185 to 210 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Thr Val Pro Ala Ser Ala Tyr Gln Val Arg Asn Ser Thr Gly Leu
1 5 10 15

Tyr His Val Thr Asn Asp Cys Pro Asn Ser
20 25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP17, amino acids 2255-2275 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ser Phe Asp Pro Leu Val Ala Glu Glu Asp Glu Arg Glu Ile Ser Val
1 5 10 15

Pro Ala Glu Ile Leu
20

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP18, amino acids 2232 to 2255 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Trp Arg Gln Glu Met Gly Gly Asn Ile Thr Arg Val Glu Ser Glu
1 5 10 15

Asn Lys Val Val Ile Leu Asp
20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3E, amino acids 1928-1951 of HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Val Ser Pro Thr His Tyr Val Pro Glu Ser Asp Ala Ala Ala Arg
1 5 10 15

Val Thr Ala Ile Leu Ser Ser Leu
20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hepatitis C Virus
(C) INDIVIDUAL ISOLATE: DP3F, amino acids 1932-1955 of the HCV polyprotein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr His Tyr Val Pro Glu Ser Asp Ala Ala Ala Arg Val Thr Ala Ile
1 5 10 15

Leu Ser Ser Leu Thr Val Thr Gln
20

IT IS CLAIMED:

1. A method for detecting the presence of hepatitis C virus (HCV) antigens in a sample, comprising

contacting the sample with at least one antibody that is reactive with an HCV antigen, where the antibody is attached to a solid support,

examining the antibody for the presence of bound HCV antigen, where said examining involves reacting the solid support with an antigen-reporter complex, where the HCV antigen competes with binding of the antigen-reporter complex to the antibody, and

detecting antigen-reporter complex that is bound to the solid support.

2. The method of claim 1, where said detecting includes quantitation of the level of reporter that remains bound to the solid support.

3. The method of claim 1, where the sample is selected from the group consisting of tissue culture medium, chimpanzee serum, and human serum.

4. The method of claim 1, where the solid support is the well of a microtiter plate.

5. The method of claim 1, where the antigen used to generate the antibody comprises a polypeptide antigen selected from the group of sequences presented as SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:14, and SEQ ID NO:16.

6. The method of claim 1, where the antigen of the antigen-reporter complex comprises a polypeptide antigen selected from the group consisting of SEQ ID

NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:14, and SEQ ID NO:16.

7. The method of claim 6, where the antigen of the antigen-reporter complex comprises the polypeptide presented as SEQ ID NO:7.

8. The method of claim 6, where two antibodies are used and the antigen of the first antigen-reporter complex comprises SEQ ID NO:7 and the antigen of the second antigen-reporter complex comprises SEQ ID NO:14.

9. The method of claim 1, where said contacting is conducted in the presence of a non-ionic surfactant.

10. The method of claim 9, where the antigen of the antigen-reporter complex comprises the polypeptide presented as SEQ ID NO:16.

11. The method of claim 1, where the reporter of the antigen-reporter complex is selected from the group consisting of enzymatic reporters, radioactive reporters, and fluorescent reporters.

12. The method of claim 11, where the reporter is the enzymatic reporter horse radish peroxidase.

13. A method for detecting the presence of hepatitis C virus (HCV) antigens in a sample, comprising

contacting the sample with at least one antibody that is reactive with the polypeptide presented as SEQ ID NO:16, where the antibody is

attached to a solid support, and said contacting takes place in the presence of a polyoxyethylene sorbitan,

examining the antibody for the presence of bound HCV antigen, where said examining involves reacting the solid support with a SEQ ID NO:16 polypeptide-reporter complex, and

detecting SEQ ID NO:16 polypeptide-reporter complex that is bound to the solid support.

14. A diagnostic kit for use in screening samples for the presence of hepatitis C virus (HCV) antigens comprising

at least one antibody that is reactive with an HCV antigen, and

an antigen-reporter complex, where the HCV antigen competes with binding of the antigen-reporter complex to the antibody.

15. The kit of claim 14, where said antibody is attached to a solid support.

16. The kit of claim 14, where the antigen of the antigen-reporter complex comprises a polypeptide antigen selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:14 and SEQ ID NO:16.

17. The kit of claim 16, where the antigen of said antigen-reporter comprises SEQ ID NO:7.

18. The kit of claim 16, where the antigen of said antigen-reporter complex comprises SEQ ID NO:14.

19. The kit of claim 16, where the antigen of said antigen-reporter complex comprises SEQ ID NO:16.

20. The kit of claim 14, where the reporter of the antigen-reporter complex is selected from the group consisting of enzymatic reporters, radioactive reporters, and fluorescent reporters.

21. Purified antibodies that are immunoreactive with a polypeptide consisting essentially of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:14 and SEQ ID NO:16.

22. The antibodies of claim 21, which are polyclonal antibodies.

23. The antibodies of claim 21, which are monoclonal antibodies.

24. A purified HCV polypeptide antigen characterized by:

(a) an epitope formed by the sequence presented as SEQ ID NO:3; and

(b) effective to recognize, by immunoreactivity with HCV-specific antibodies in human HCV anti-sera, a substantially broader range of HCV anti-sera than is recognized by a 362 amino acid HCV peptide having a 14 amino acid N-terminal region amino acid overlap with the SEQ ID NO:3.

25. The antigen of claim 24, whose N-terminal amino acid region of overlap with said 362 amino

acid HCV peptide is substantially no greater than 14 amino acids.

26. The antigen of claim 24, which includes the amino acid sequence of SEQ ID NO:3.

27. A purified polypeptide antigen consisting essentially of amino acid residues contained in one from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:14 and SEQ ID NO:16.

28. A diagnostic kit for use in screening human blood containing antibodies specific against hepatitis C virus (HCV) infection comprising

an HCV polypeptide antigen characterized by (a) an epitope formed by the sequence presented as SEQ ID NO:3; and (b) effective to recognize, by immunoreactivity with HCV-specific antibodies in human HCV anti-sera, a substantially broader range of HCV anti-sera than is recognized by a 362 amino acid HCV peptide having a 14 amino acid N-terminal region amino acid overlap with the SEQ ID NO:3; and

means for detecting the binding of said antibodies to the antigen.

29. The kit of claim 28, wherein the N-terminal amino acid region of overlap between said antigen and said 362 amino acid HCV peptide is substantially no greater than 14 amino acids.

30. The kit of claim 28, wherein said detecting means includes a solid support to which said antigen is attached, and a reporter-labeled anti-human antibody effective to bind to human HCV-

specific antibodies, with such bound to said antigen.

31. A method of detecting hepatitis C virus (HCV) infection in an individual comprising

reacting serum from an HCV-infected test individual with an HCV polypeptide antigen characterized by (a) an epitope formed by the sequence presented as SEQ ID NO:3; and (b) effective to recognize, by immunoreactivity with HCV-specific antibodies in human HCV anti-sera, a substantially broader range of HCV anti-sera than is recognized by a 362 amino acid HCV peptide having a 14 amino acid N-terminal region amino acid overlap with the SEQ ID NO:3; and

examining the antigen for the presence of bound antibody.

32. The method of claim 31, wherein the peptide antigen is attached to a solid support, said reacting includes reacting the peptide antigen with the support, and subsequently reacting the support with a reporter-labeled anti-human antibody, and said examining includes detecting the presence of reporter-labeled antibody on the solid support.

33. A method for detecting the presence of hepatitis C virus (HCV) antigens in the presence of HCV antibodies in a sample obtained from an HCV-infected host, comprising

generating at least one antibody capabable of binding an HCV antigen that is not normally immunoreactive with sera from the HCV-infected host,

contacting the sample with said antibody under conditions that promote the binding of the antibody

and the HCV antigen that is not normally immunoreactive with sera from the HCV-infected host, where the antibody is attached to a solid support,

examining the antibody for the presence of bound HCV antigen, where said examining involves reacting the solid support with an antigen-reporter complex, where said HCV antigen competes with binding of the antigen-reporter complex to the antibody, and

detecting antigen-reporter complex that is bound to the solid support.

34. The method of claim 33, wherein said conditions that promote the binding of the antibody and the HCV antigen that is not normally immunoreactive with sera from the HCV-infected host include the addition of a non-ionic surfactant.

35. The method of claim 34, wherein said surfactant is a polyoxyethylene sorbitan.

36. The method of claim 34, where said HCV antigen that is not normally immunoreactive with sera from the HCV-infected host contains the polypeptide sequence presented as SEQ ID NO:16.

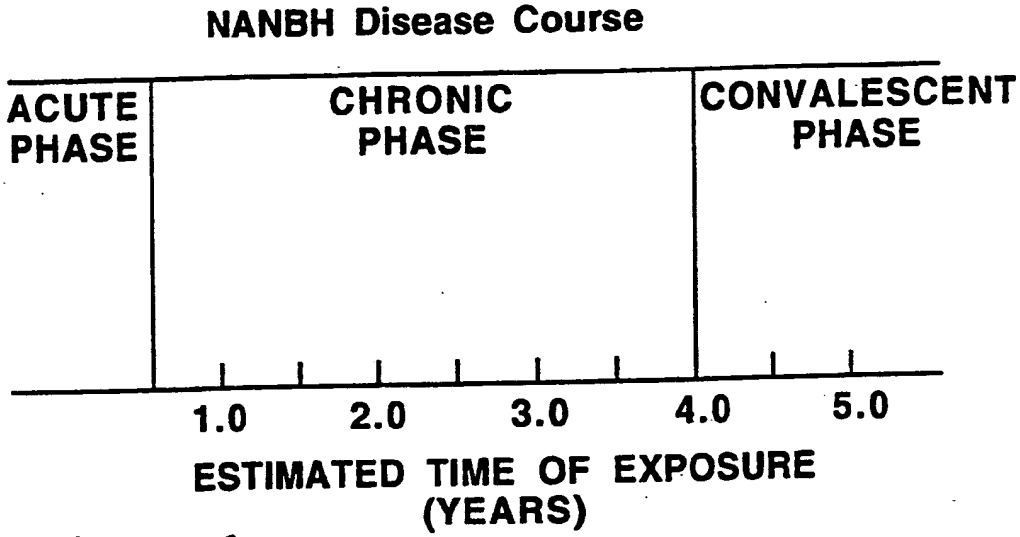

NANBH Disease Course
ACUTE PHASE
CHRONIC PHASE
CONVALESCENT PHASE
1.0
2.0
3.0
4.0
5.0
ESTIMATED TIME OF EXPOSURE (YEARS)

Fig. 1

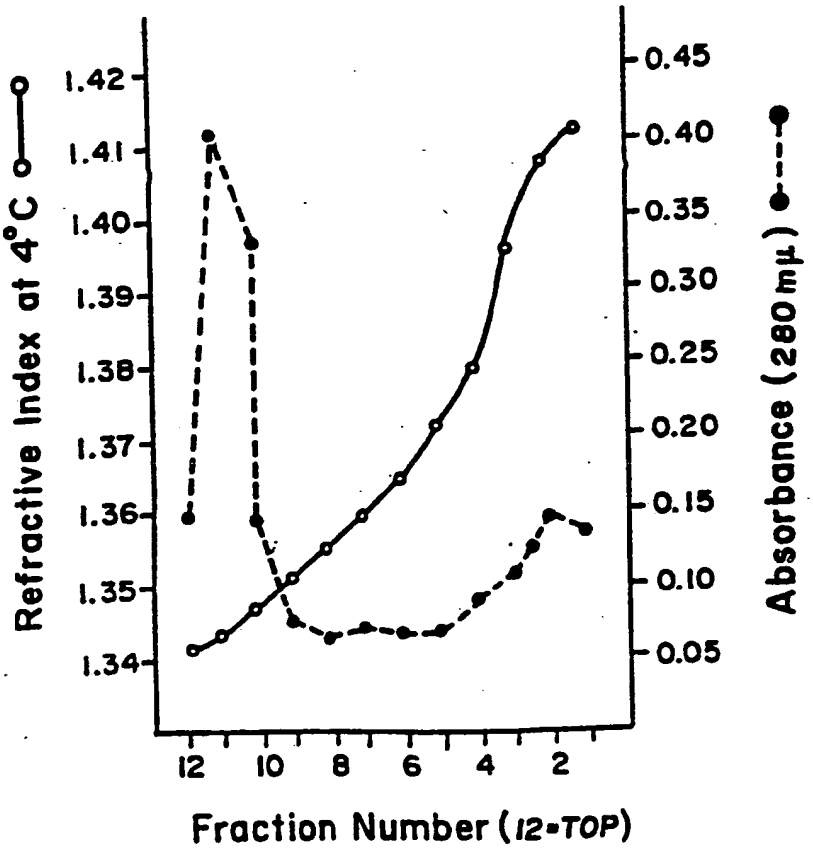

Refractive Index at 4°C
1.42
1.41
1.40
1.39
1.38
1.37
1.36
1.35
1.34
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Absorbance (280 mμ)
12
10
8
6
4
2
Fraction Number (12=TOP)

Fig. 2

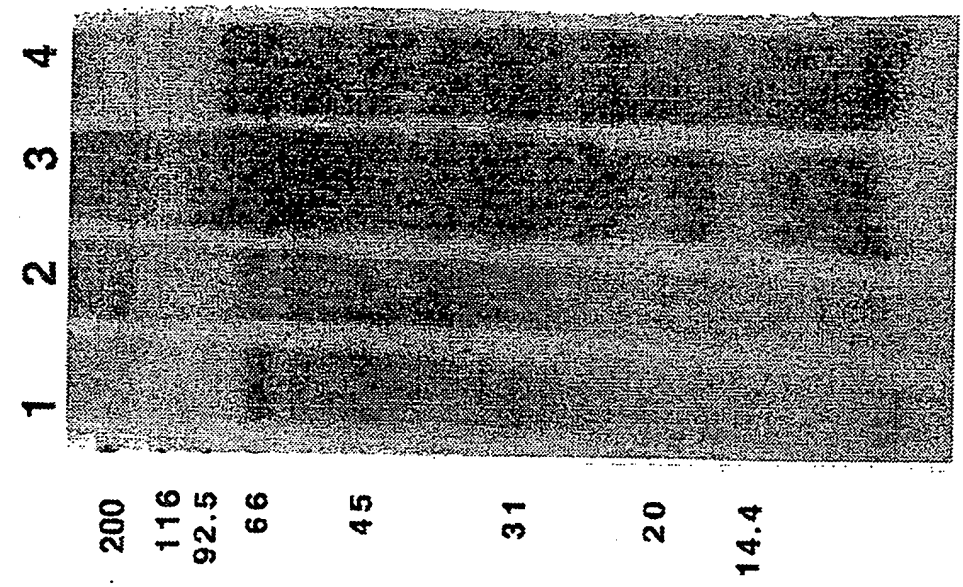

1
2
3
4
200
116
92.5
66
45
31
20
14.4

Fig. 4

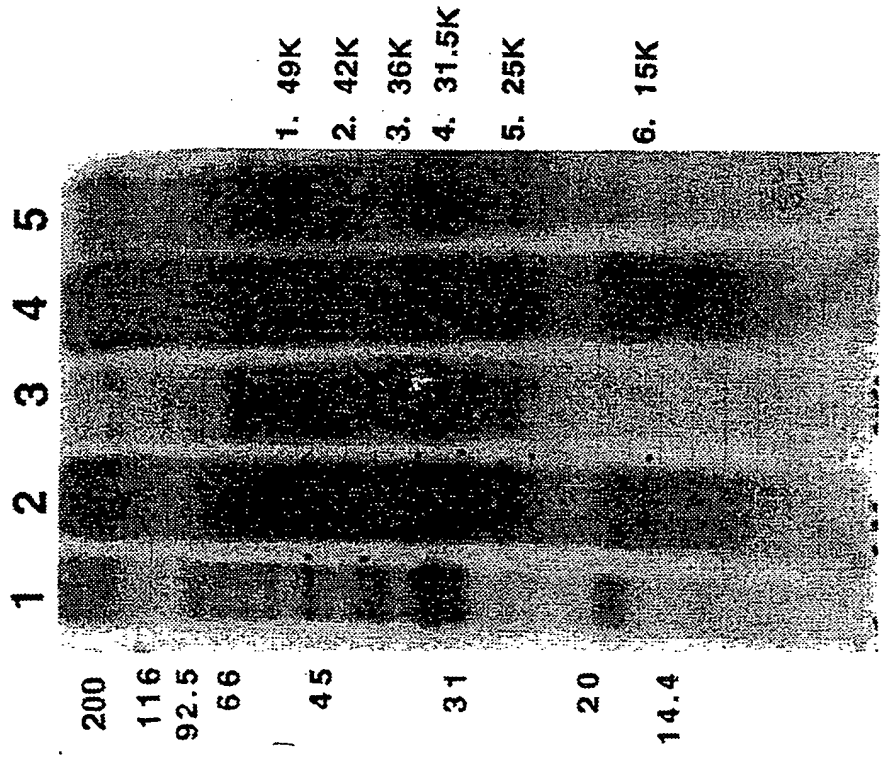

1. 49K
2. 42K
3. 36K
4. 31.5K
5. 25K
6. 15K
1
2
3
4
5
200
116
92.5
66
45
31
20
14.4

Fig. 3

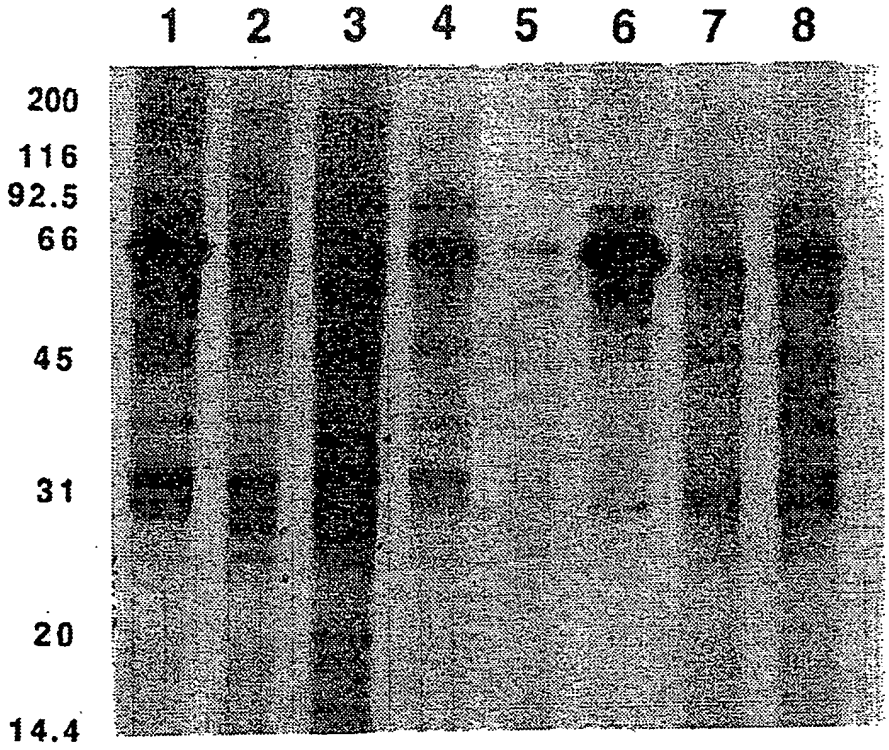



Fig. 5

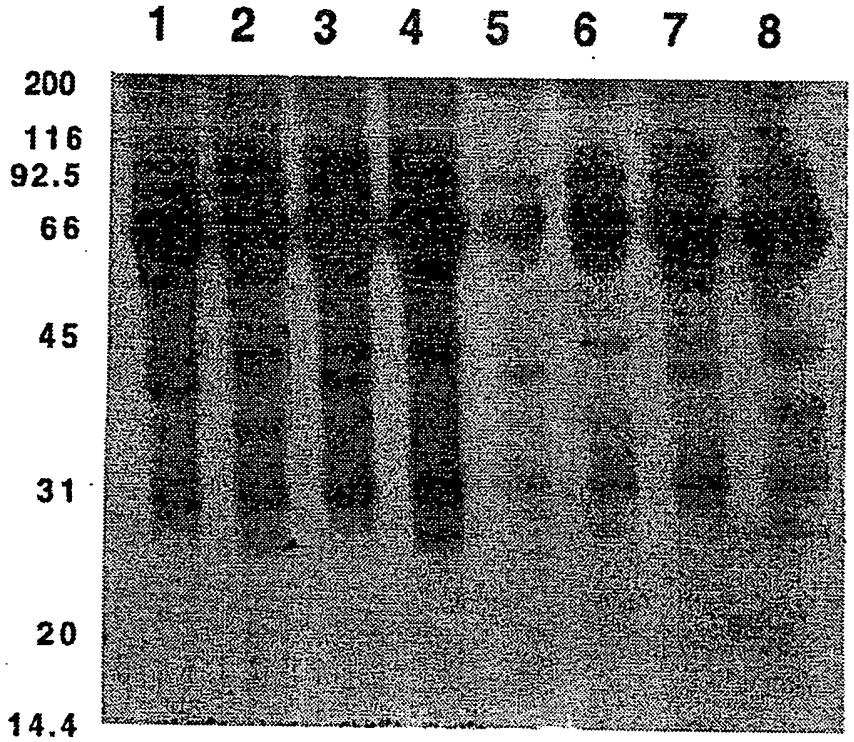



Fig. 6

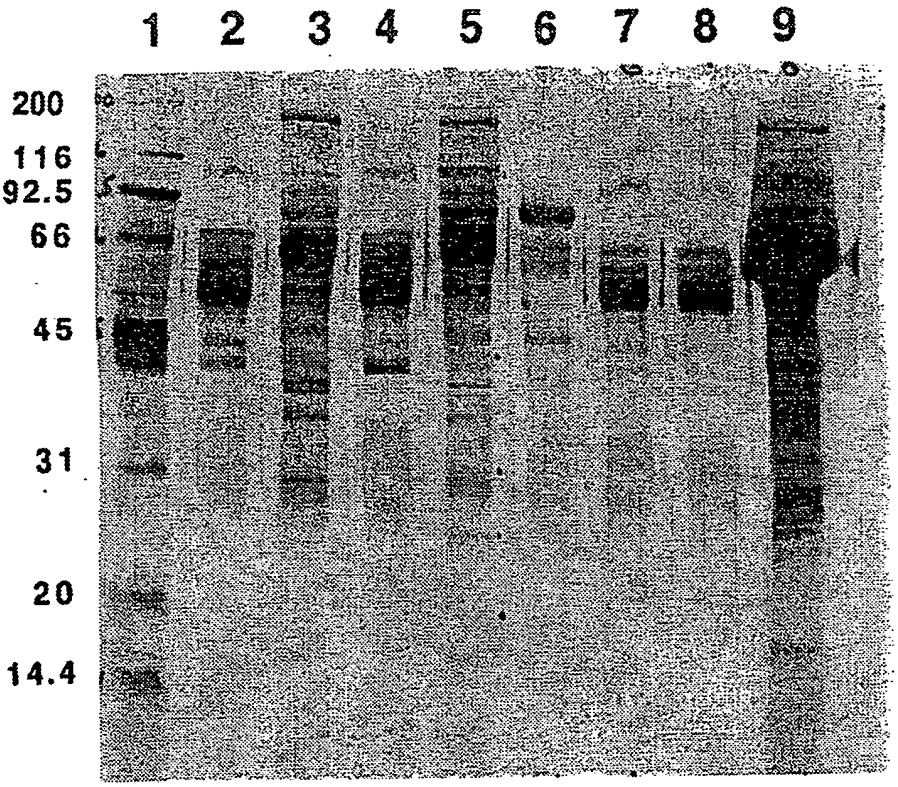



Fig. 7

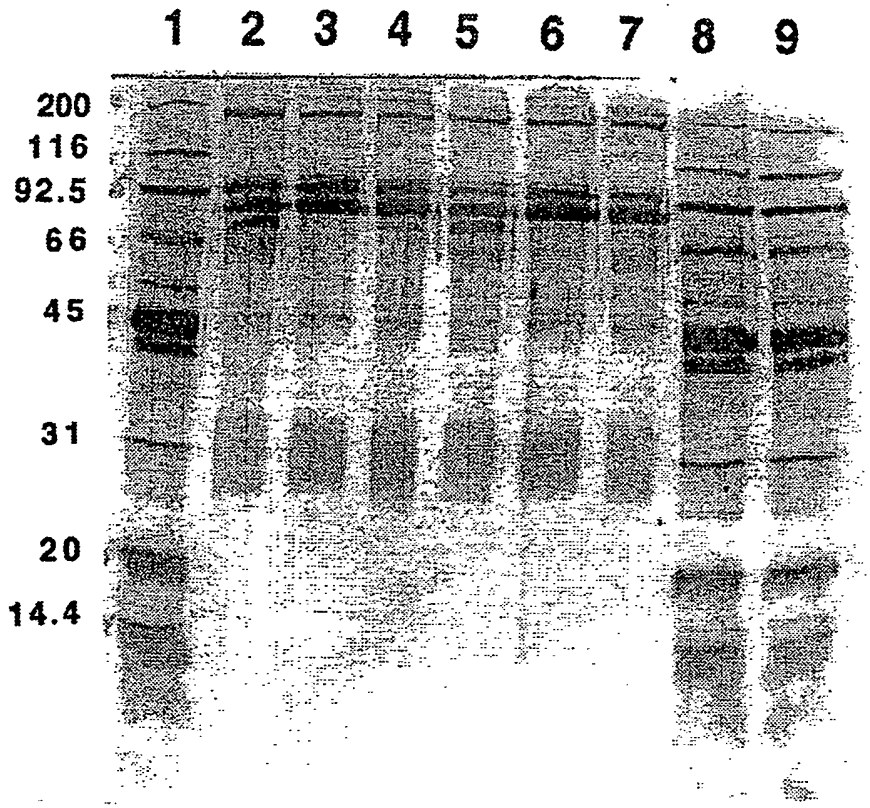



Fig. 8

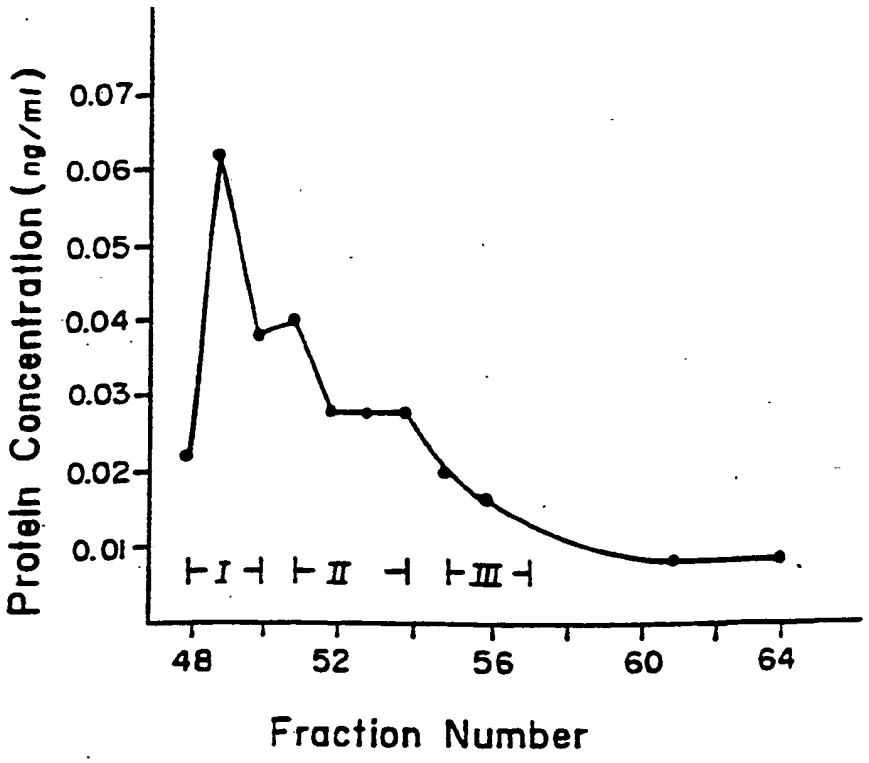

Protein Concentration (ng/ml)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
I
II
III
48
52
56
60
64
Fraction Number

Fig. 9

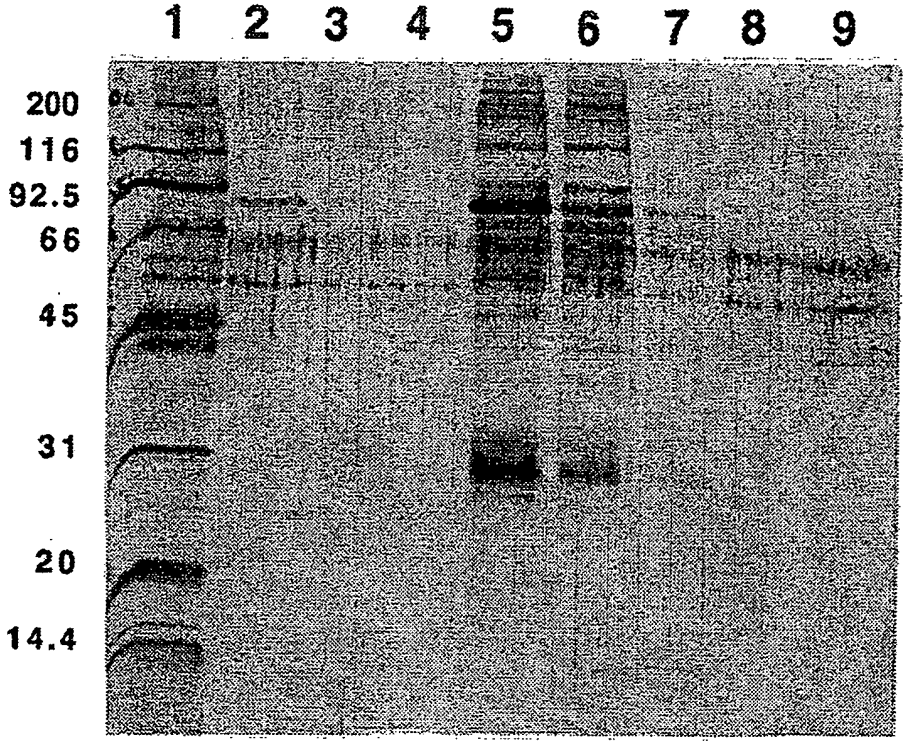



Fig. 10

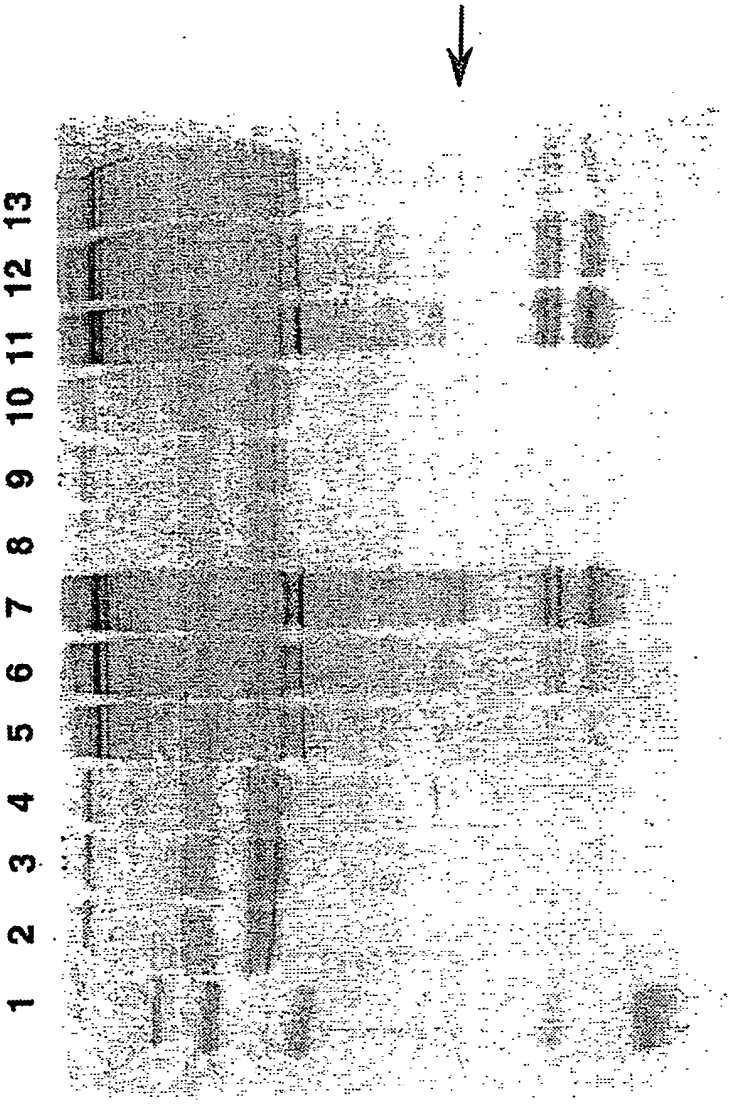



Fig. 11

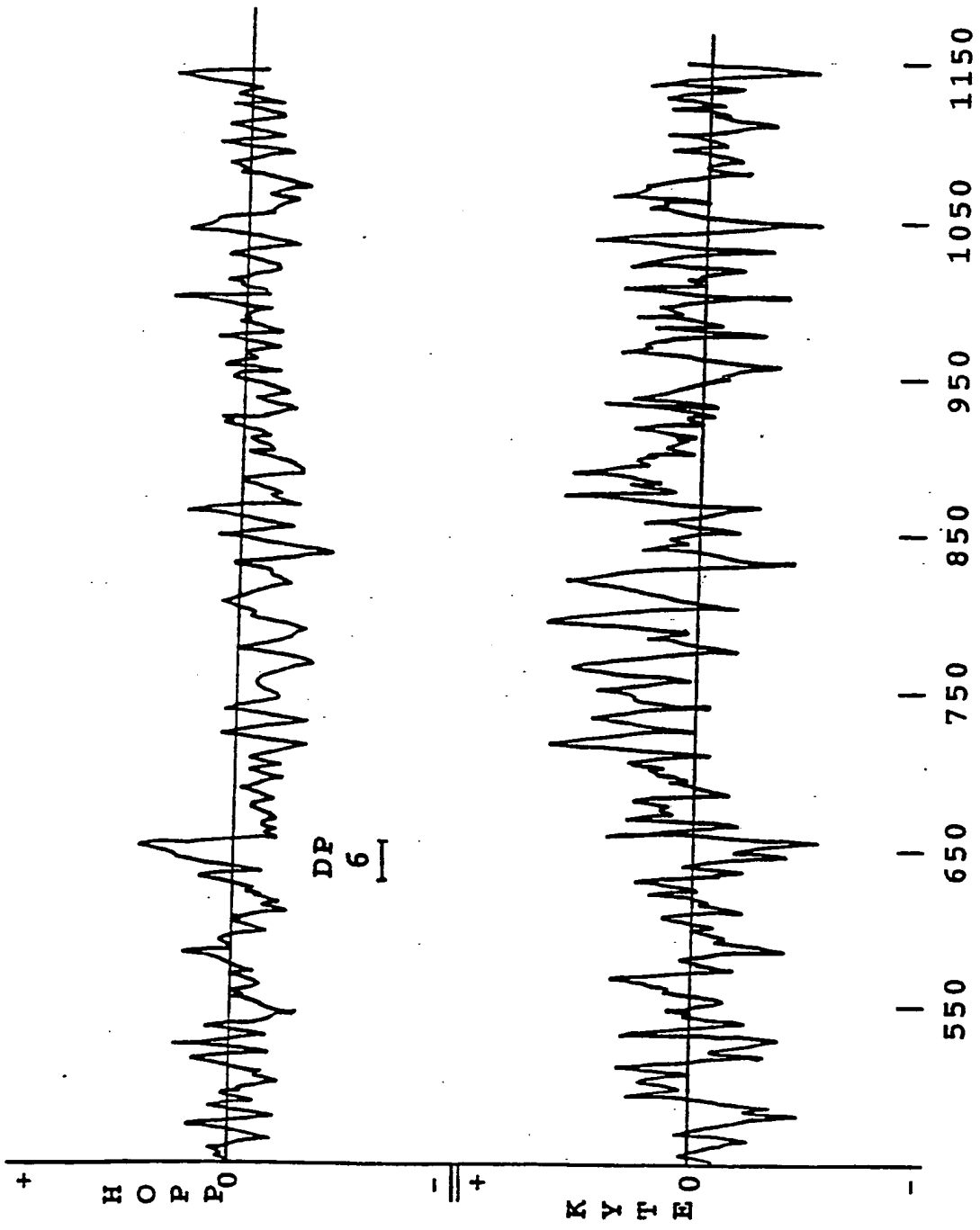



Fig. 12A

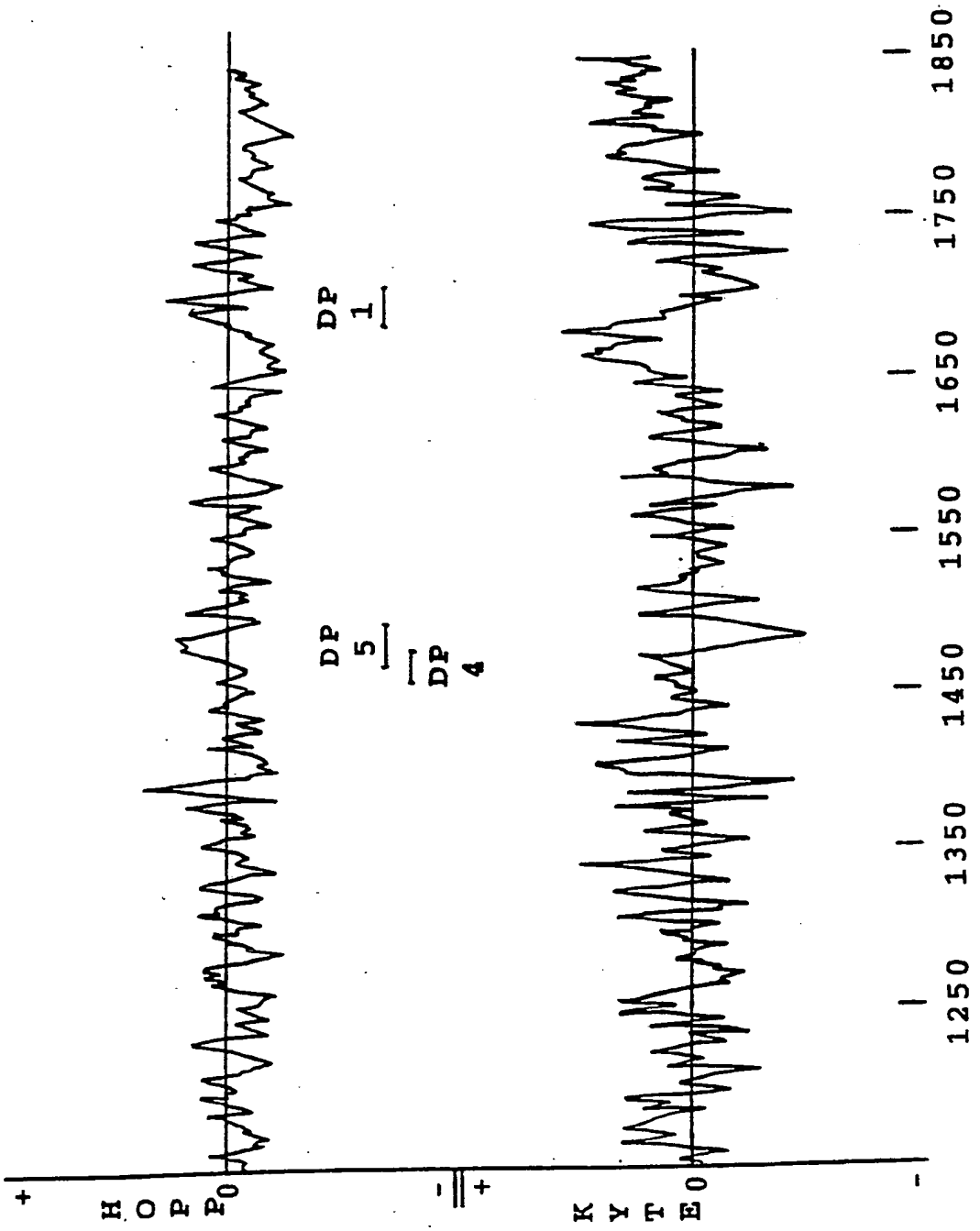



Fig. 12A con't

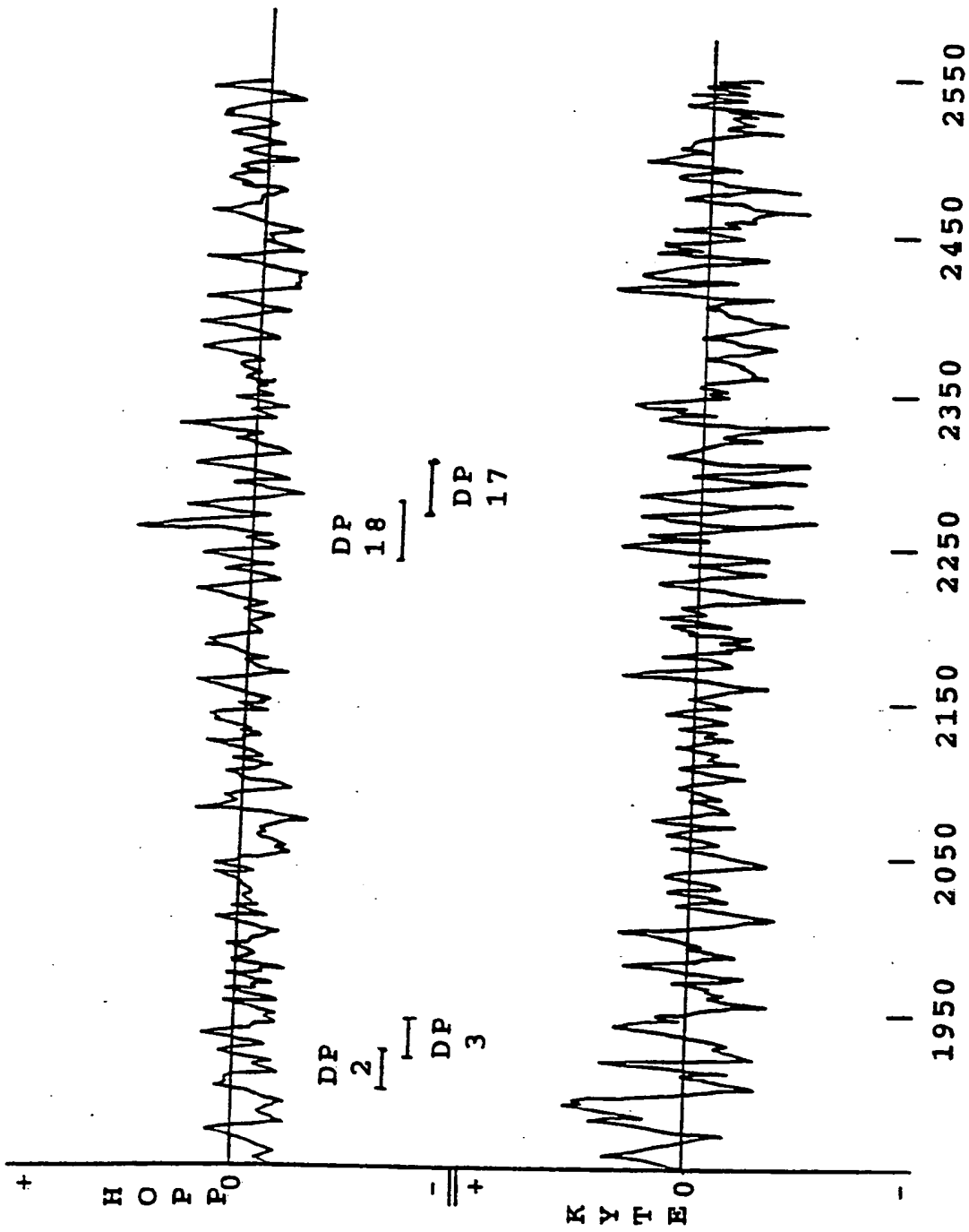

HOP
KYTE
+
-
0
DP 2
DP 3
DP 18
DP 17
1950
2050
2150
2250
2350
2450
2550

Fig. 12B

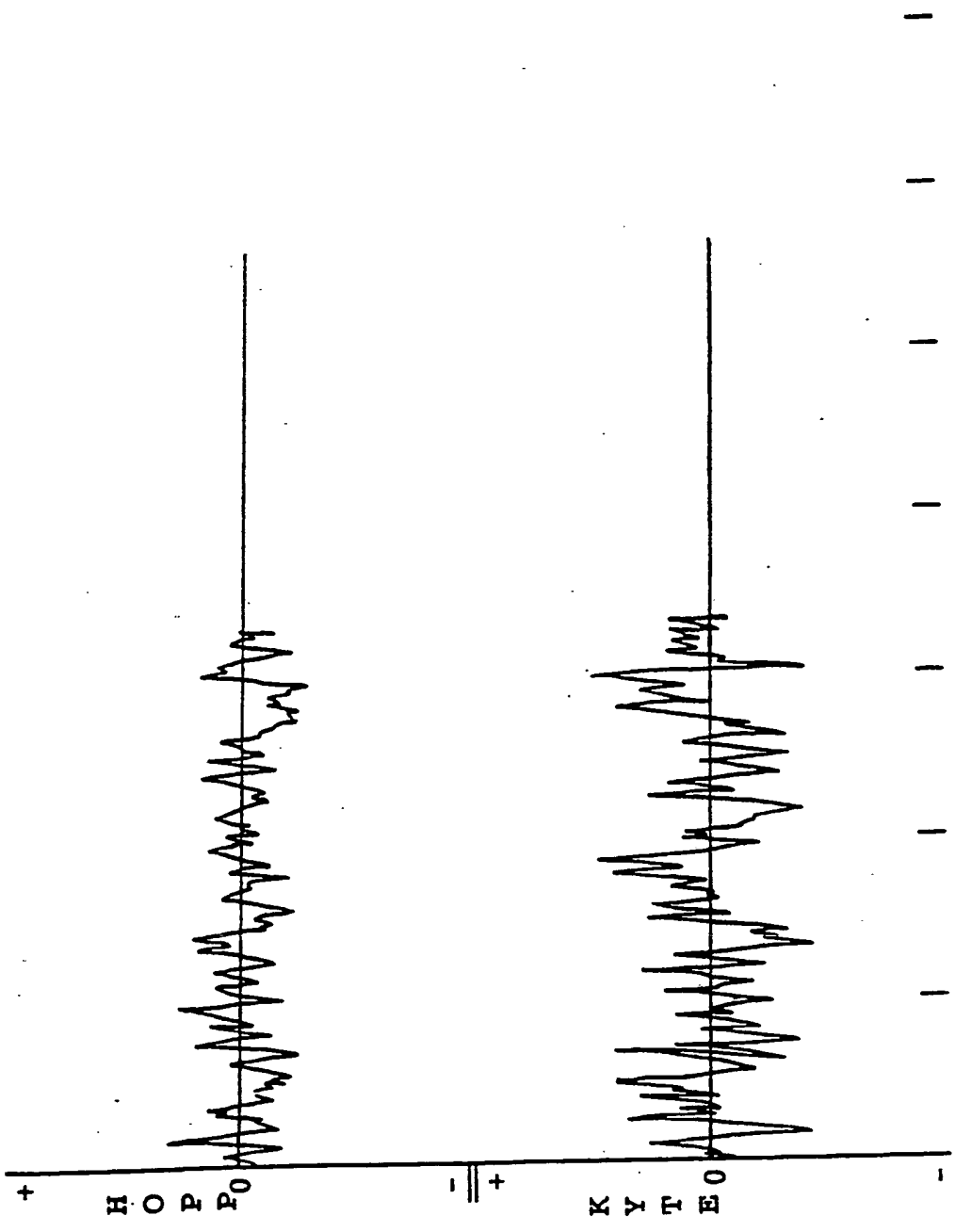



Fig. 12b con't

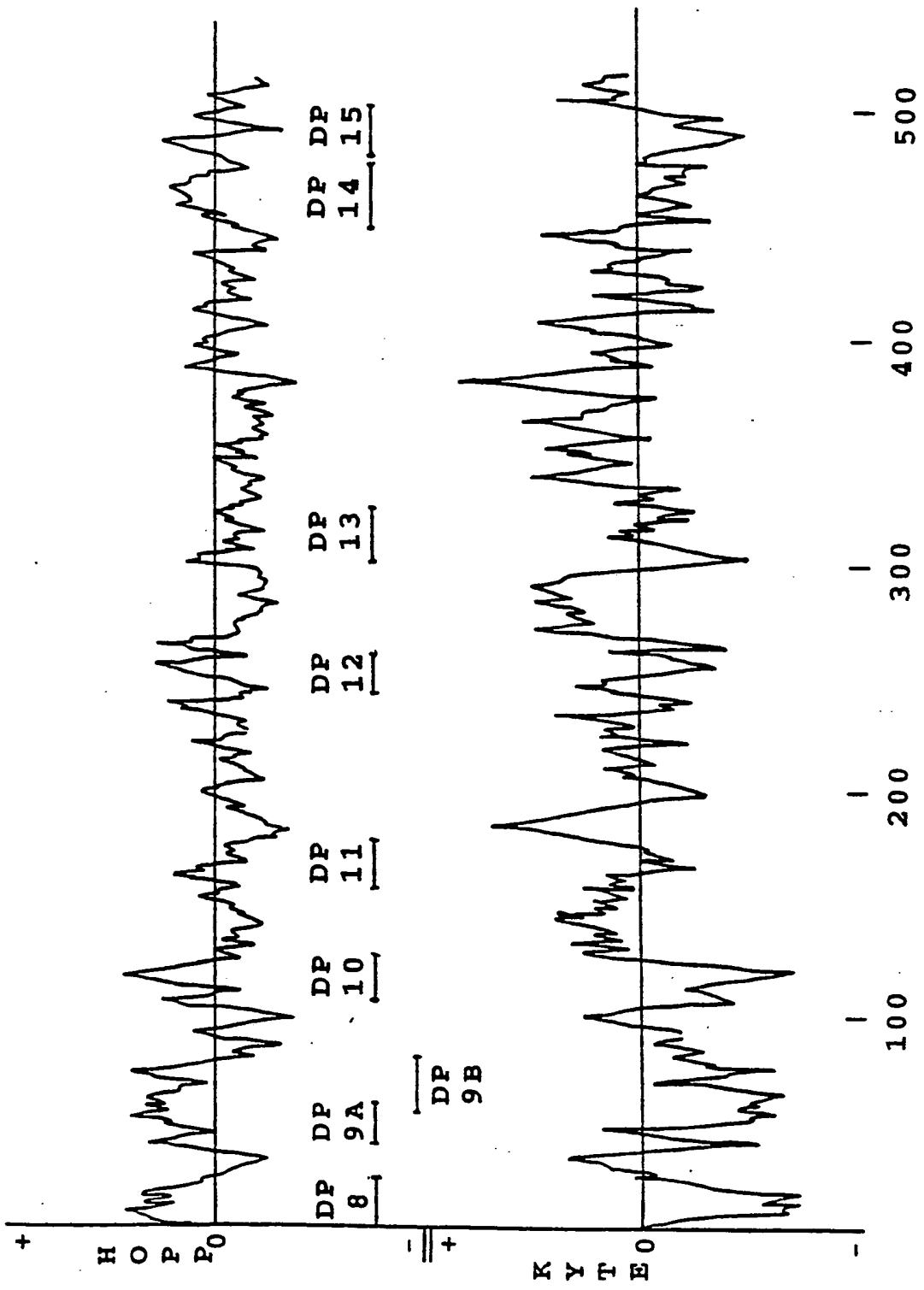

DP 8
DP 9A
DP 9B
DP 10
DP 11
DP 12
DP 13
DP 14
DP 15
HOP
KYTE
+
0
-
100
200
300
400
500

Fig. 12C

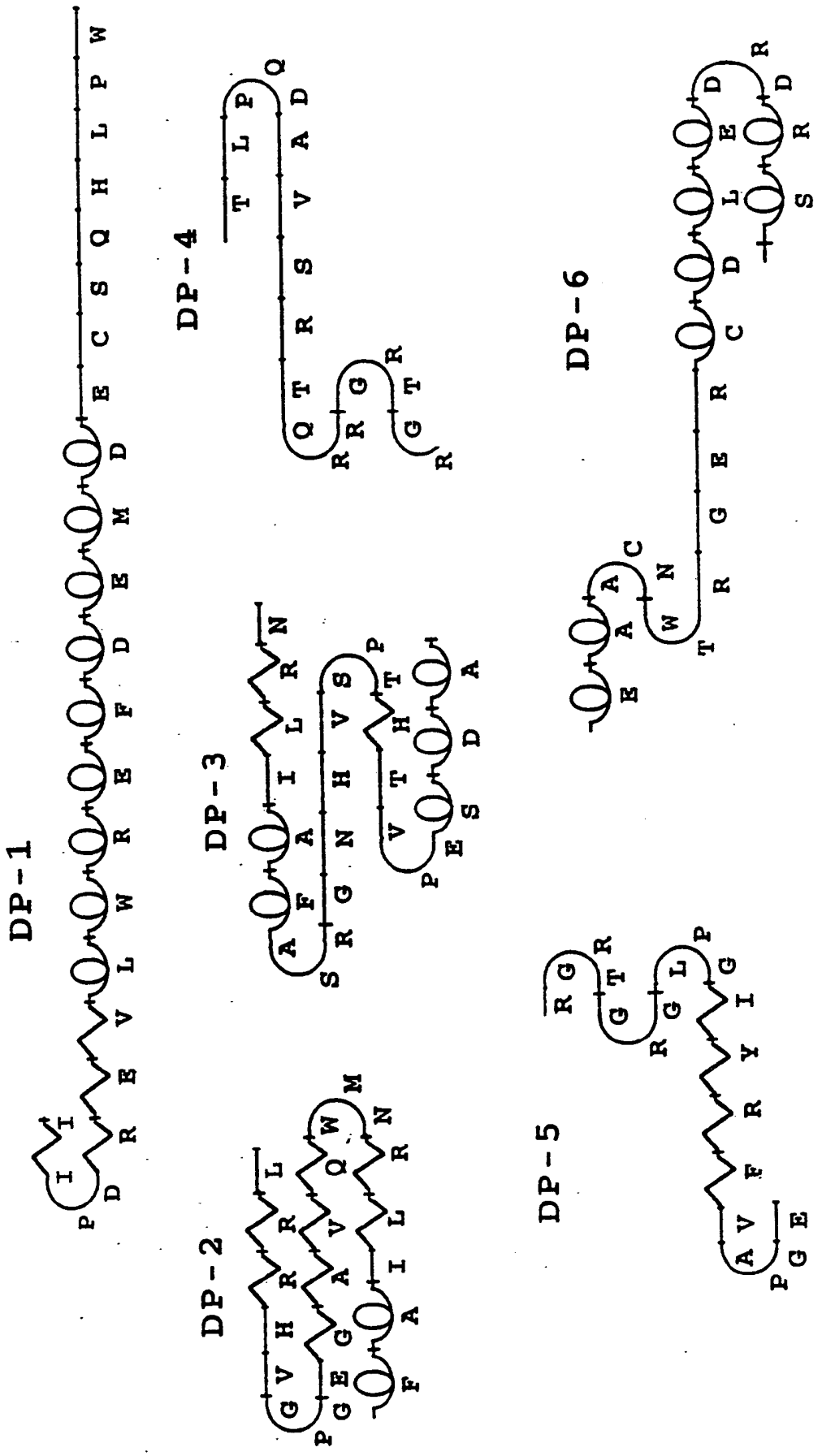



Fig. 13A

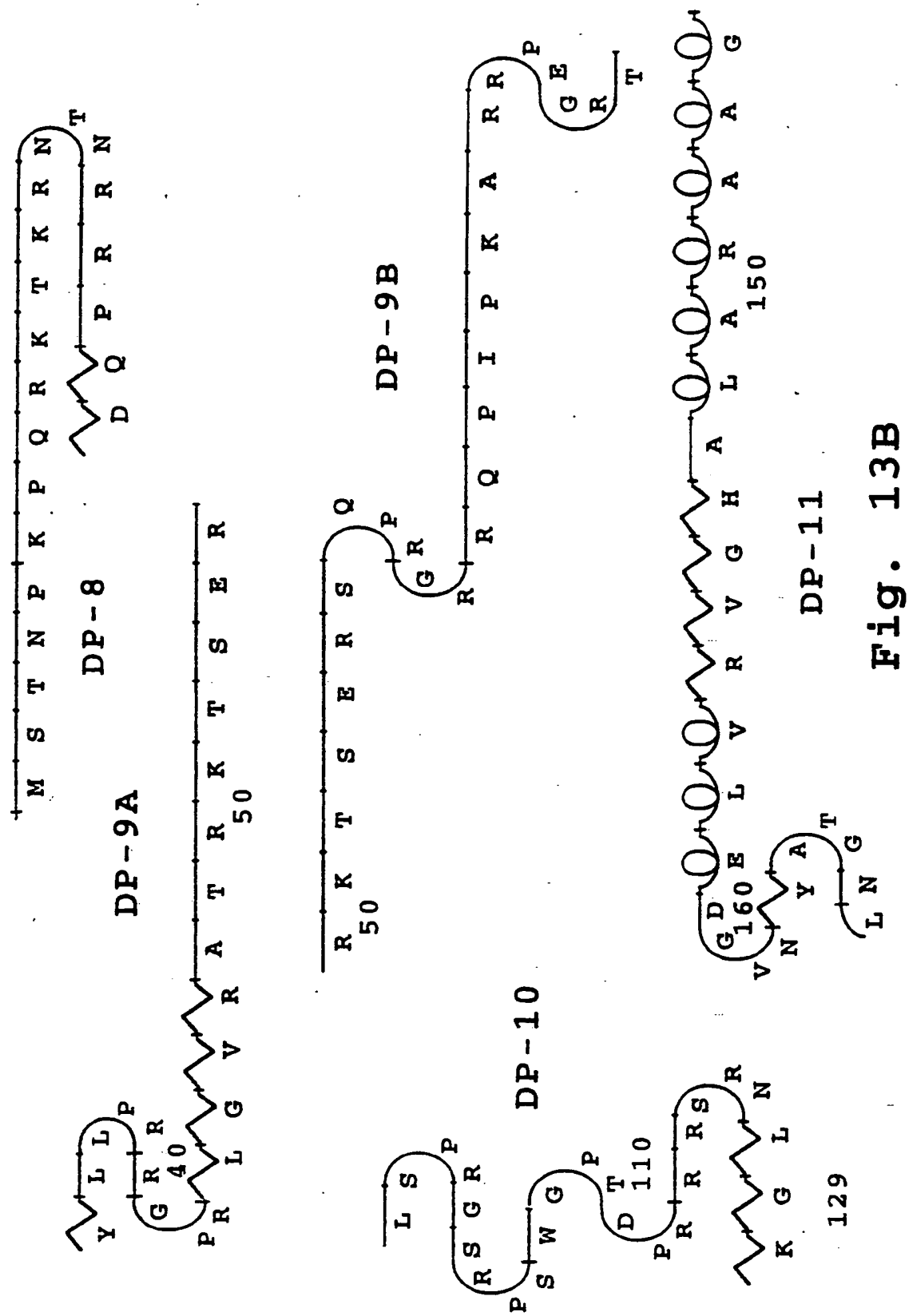

DP-8
M S T N P K P Q R K T K K R N T
D Q P R R N
DP-9A
Y L L P G R R 40 P R L G V R A T R K T S E R
50
DP-9B
R K T S E R S Q P G R R Q P I P K A R R P G E R T
50
DP-10
L S P R S G R P S W G P D T 110 P R R S R K G L N
129
DP-11
V G D E L V R V G H A L A R A A G N Y A T L N G
160
150

Fig. 13B

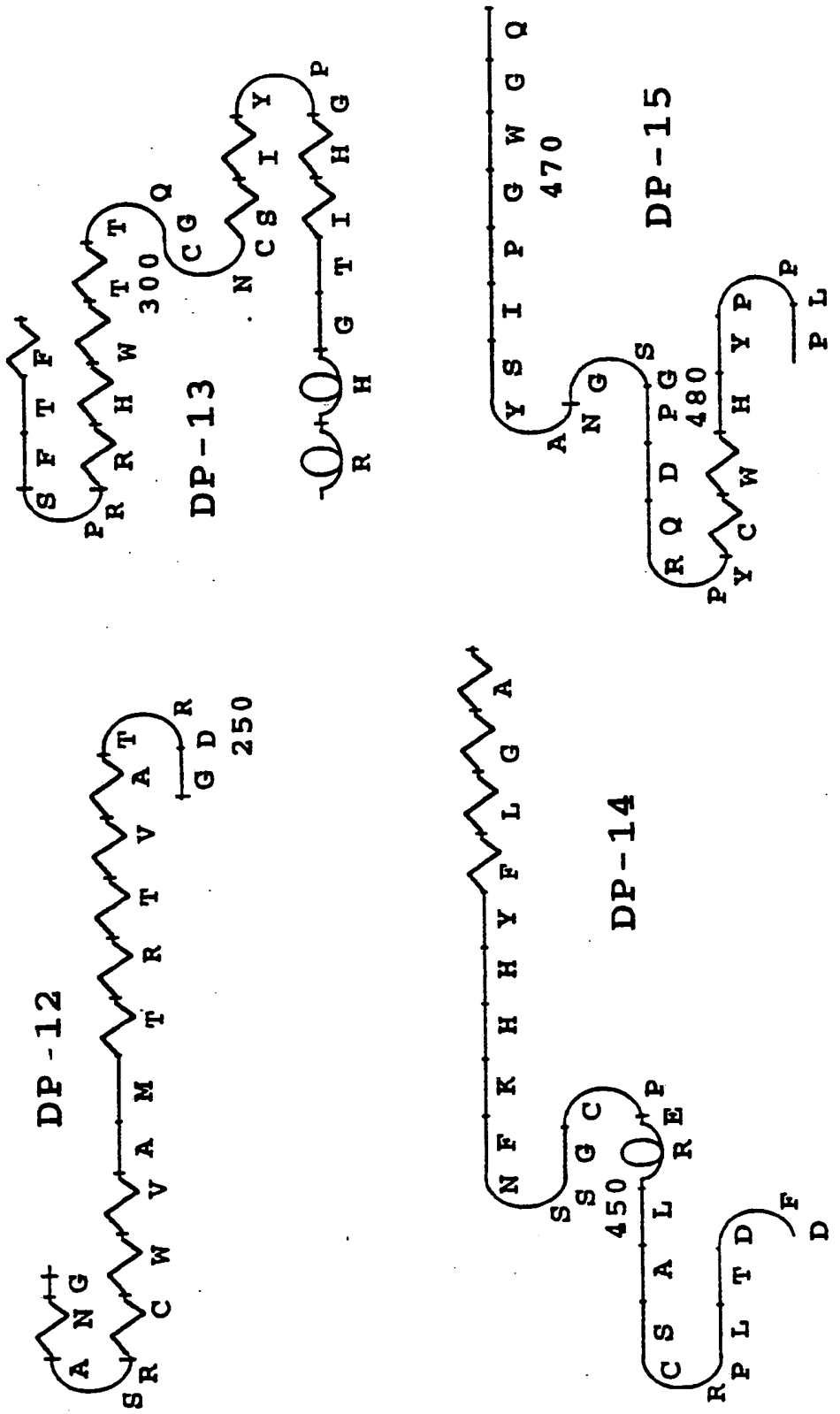



Fig. 13B con't

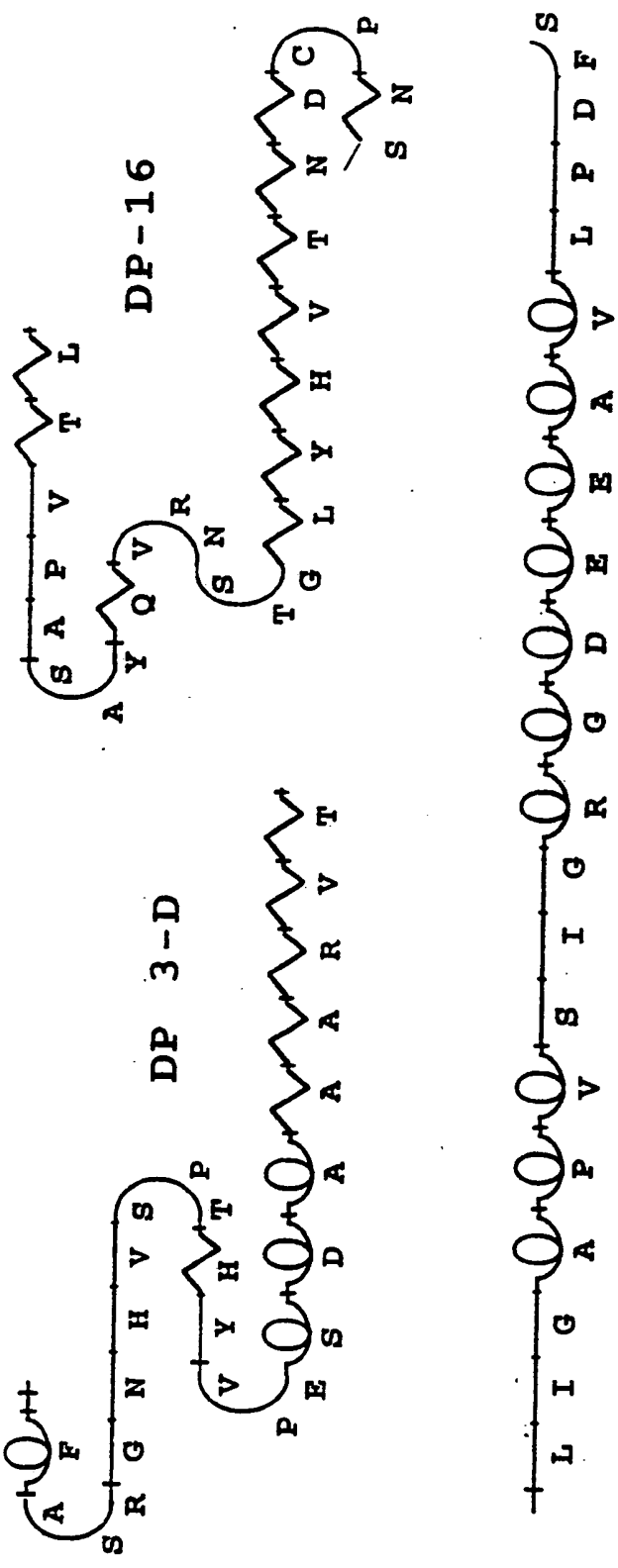



Fig. 13C

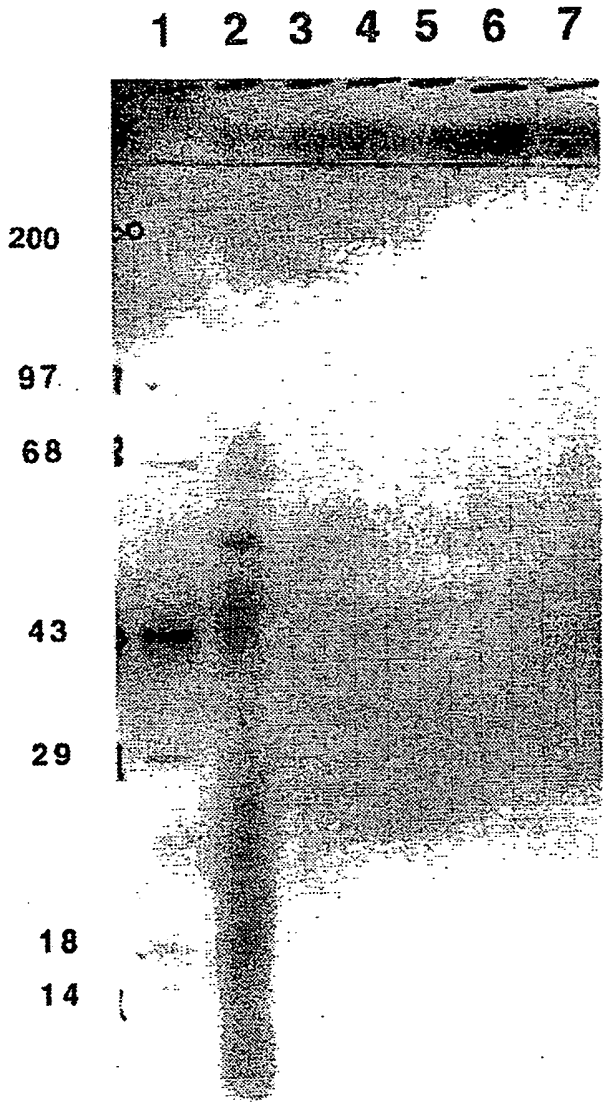



Fig. 14

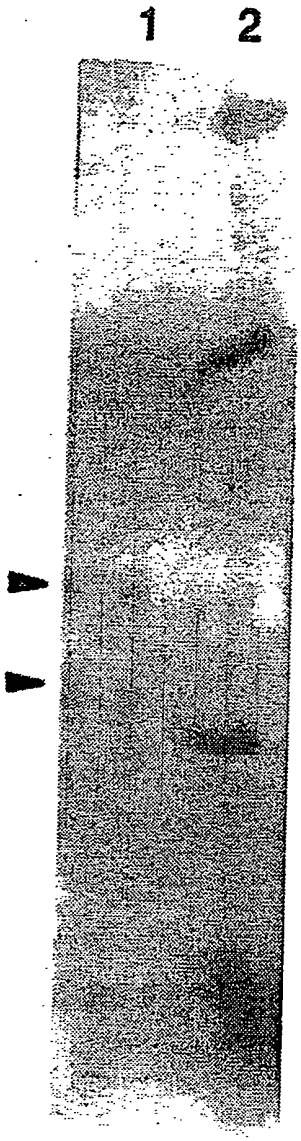
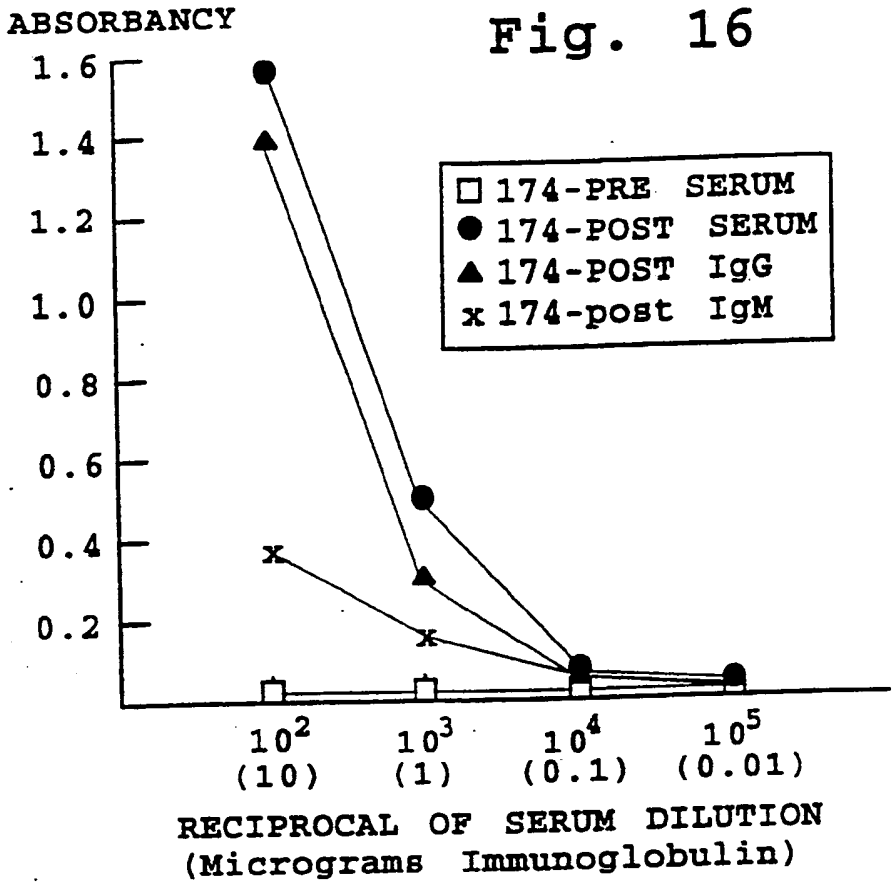



Fig. 15

Fig. 16


ABSORBANCY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
□ 174-PRE SERUM
● 174-POST SERUM
▲ 174-POST IgG
x 174-post IgM
10^2 (10)
10^3 (1)
10^4 (0.1)
10^5 (0.01)
RECIPROCAL OF SERUM DILUTION
(Micrograms Immunoglobulin)

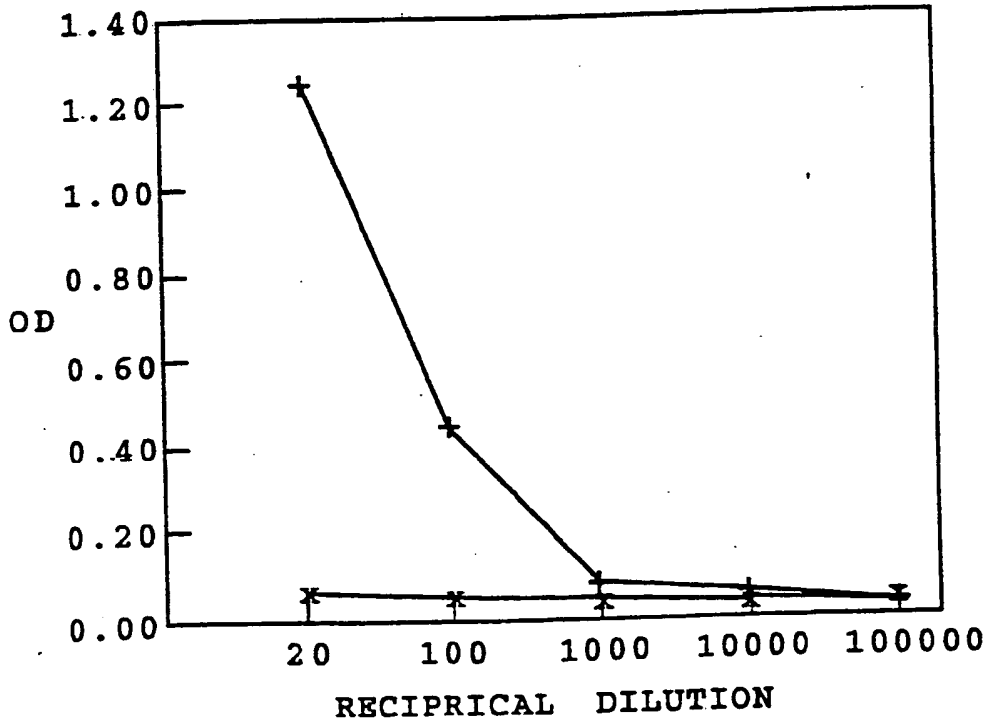

1.40
1.20
1.00
0.80
OD
0.60
0.40
0.20
0.00
20
100
1000
10000
100000
RECIPRICAL DILUTION

Fig. 17

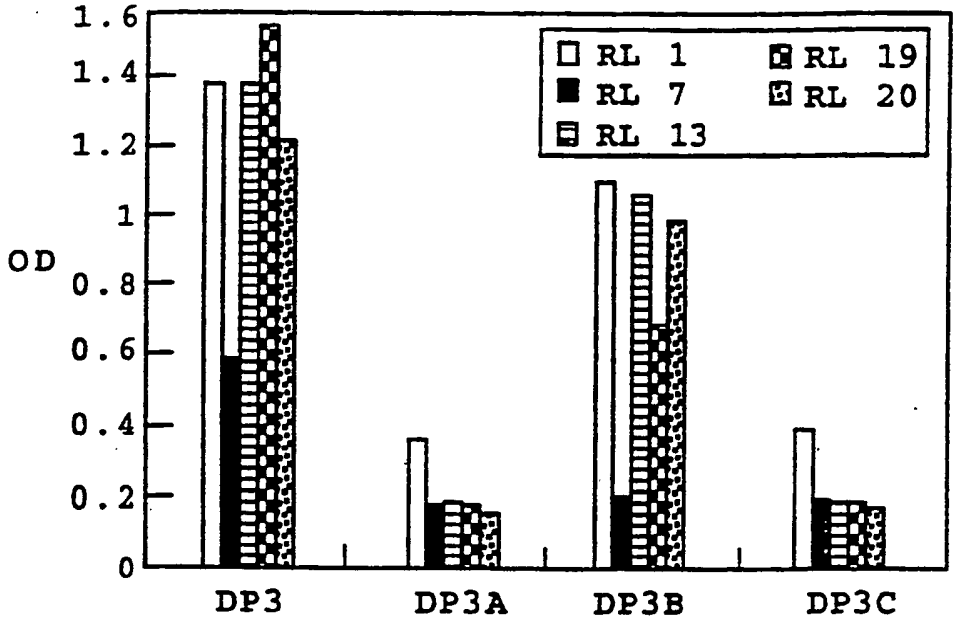

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD
RL 1
RL 7
RL 13
RL 19
RL 20
DP3
DP3A
DP3B
DP3C

Fig. 18

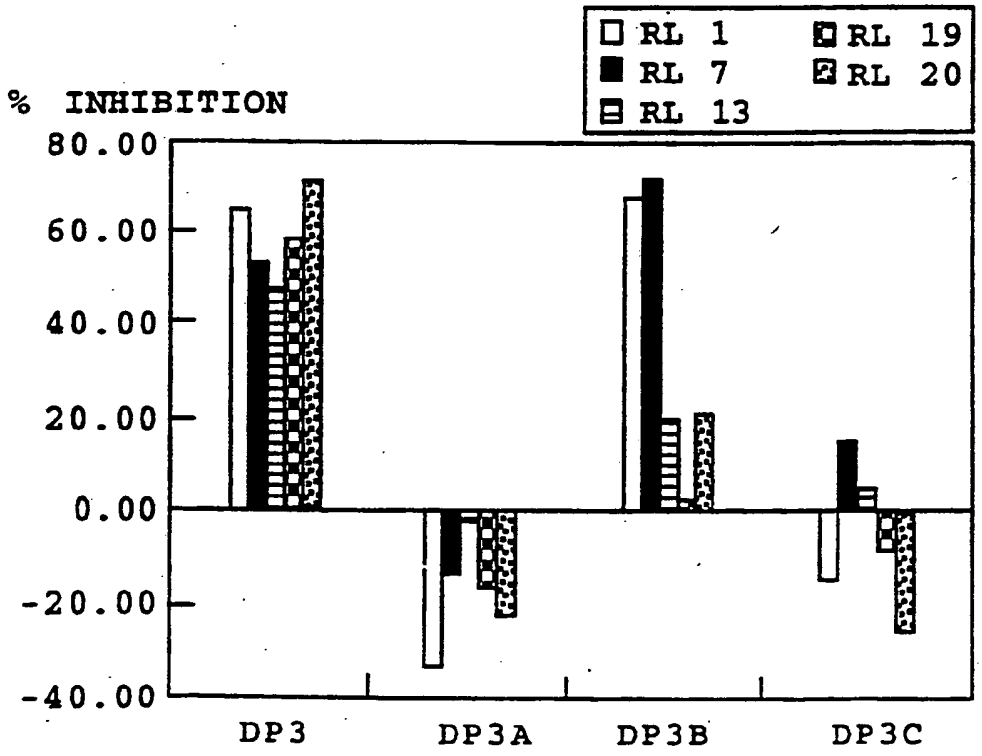
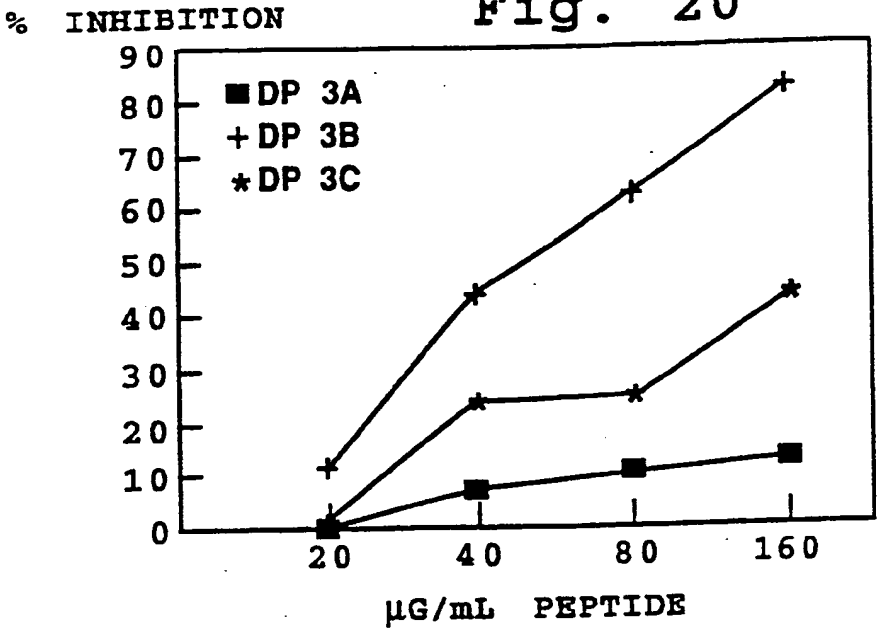

% INHIBITION
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
RL 1
RL 7
RL 13
RL 19
RL 20
DP3
DP3A
DP3B
DP3C

Fig. 19

Fig. 20


% INHIBITION
90
80
70
60
50
40
30
20
10
0
■DP 3A
+DP 3B
*DP 3C
20
40
80
160
µG/mL PEPTIDE

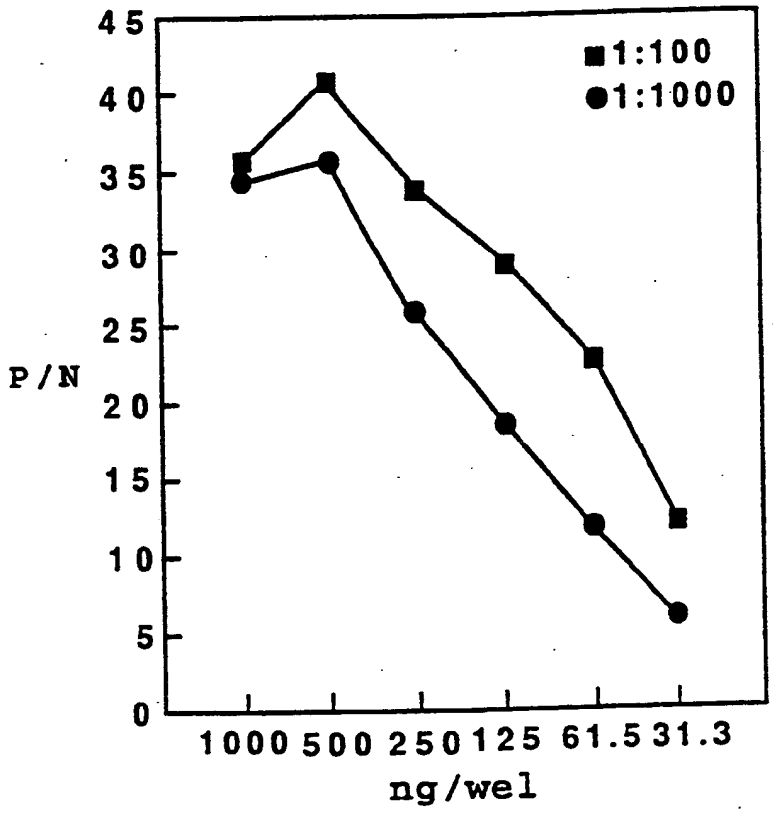

45
40
35
30
25
20
15
10
5
0
P/N
■1:100
●1:1000
1000 500 250 125 61.5 31.3
ng/wel

Fig. 21

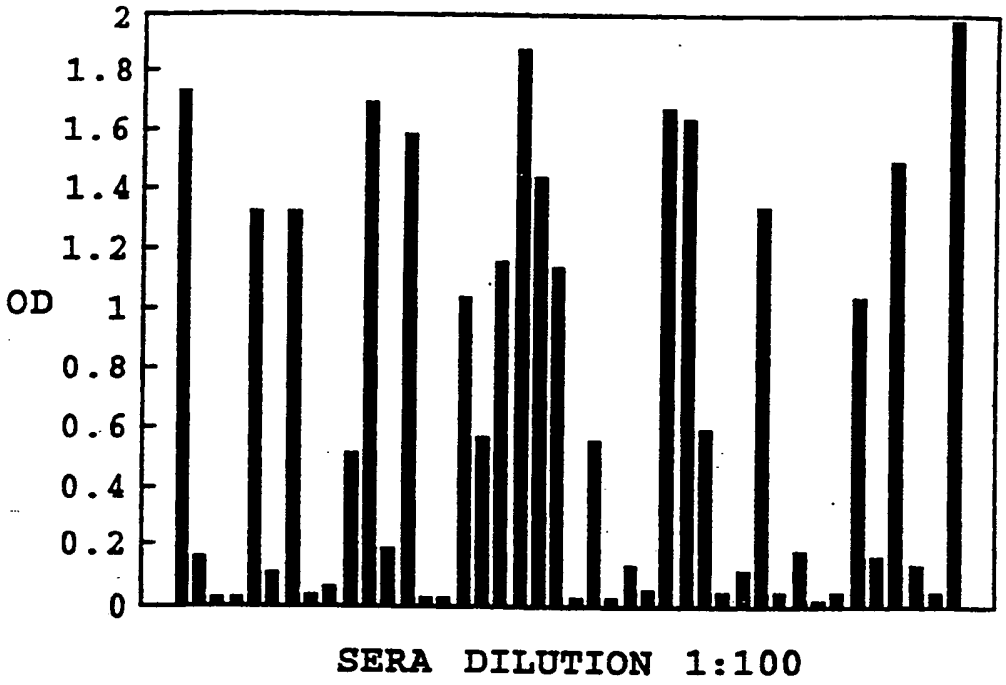



Fig. 22

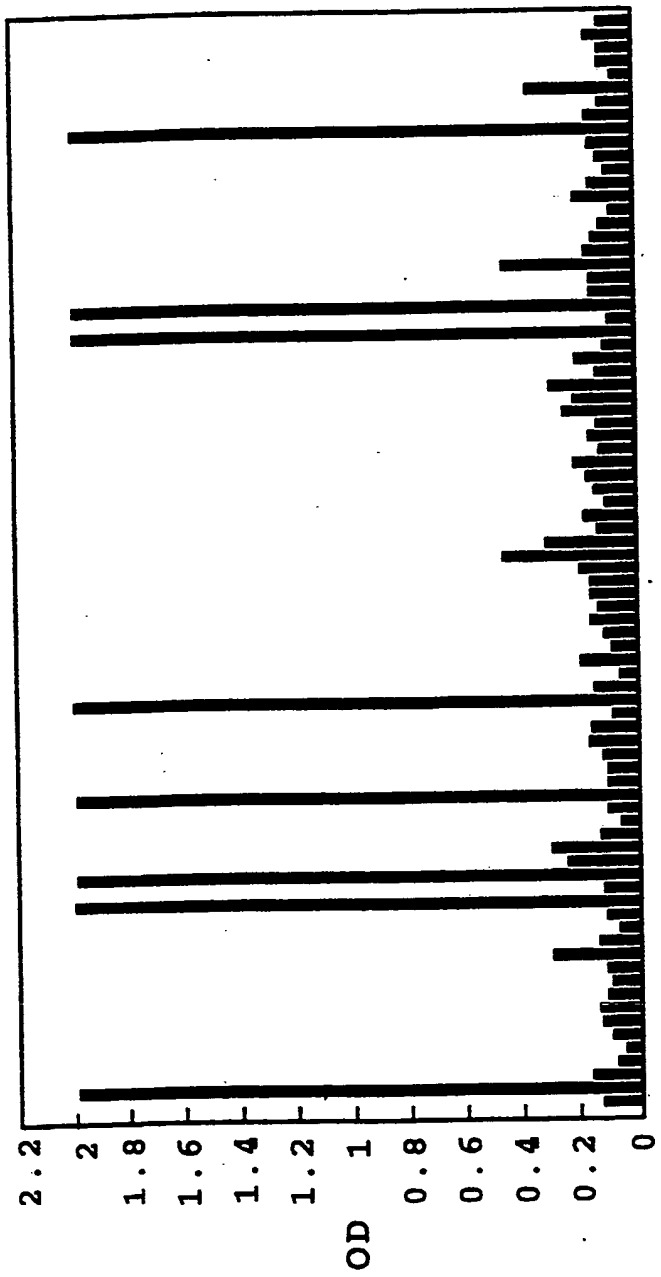



Fig. 23

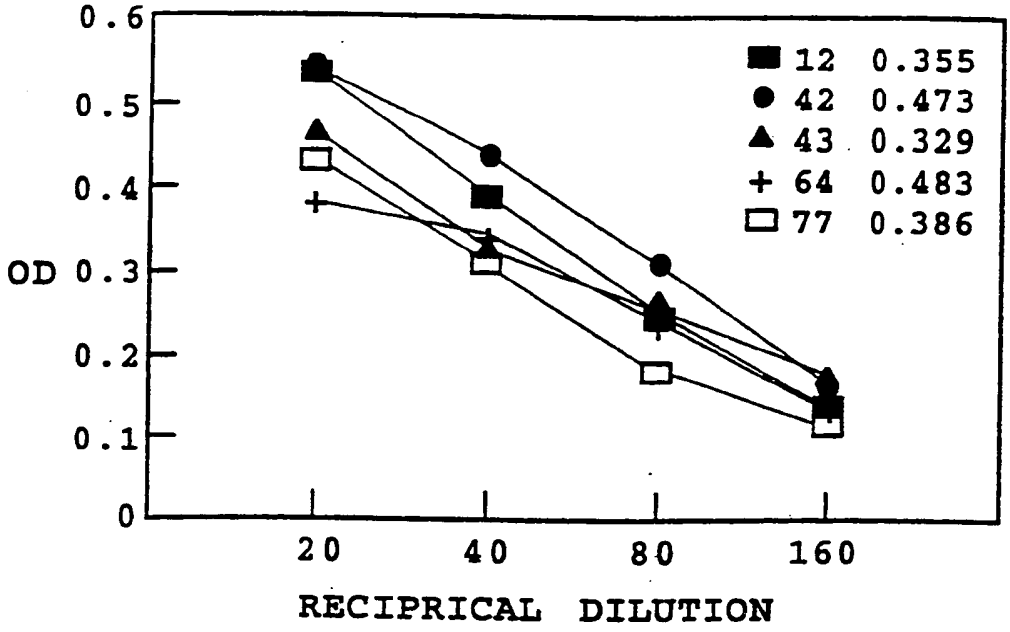



Fig. 24A

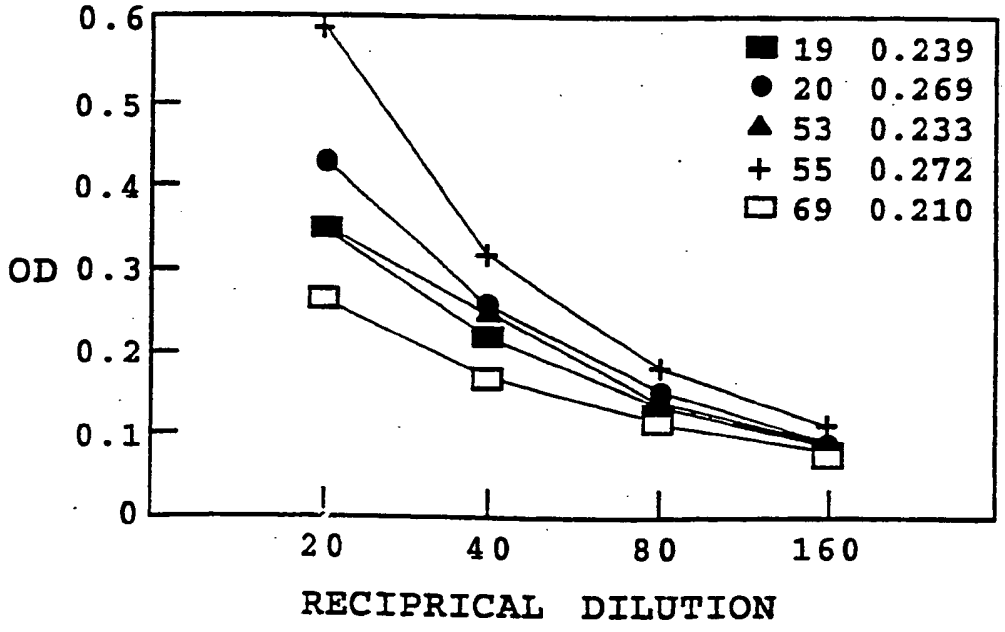



Fig. 24B

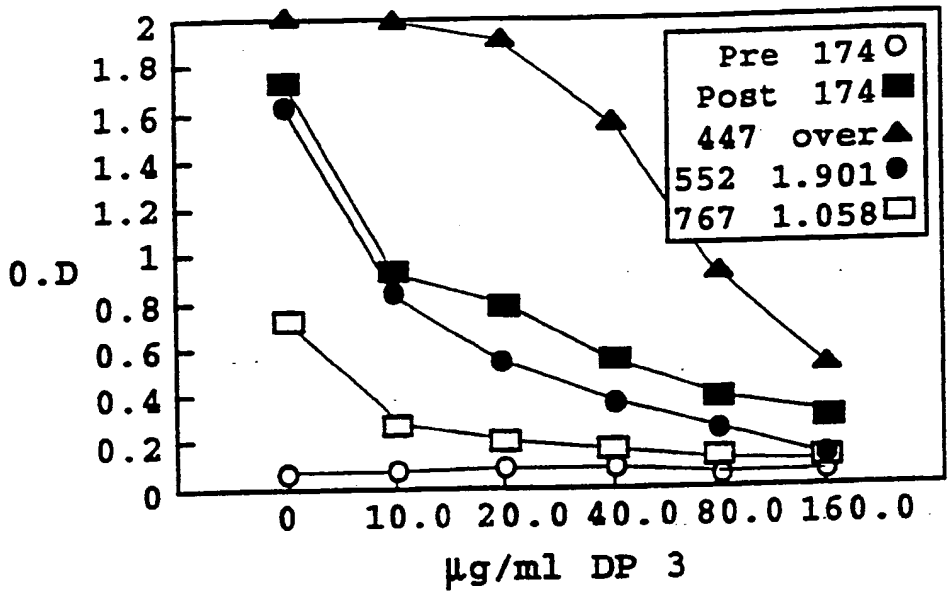



Fig.25A

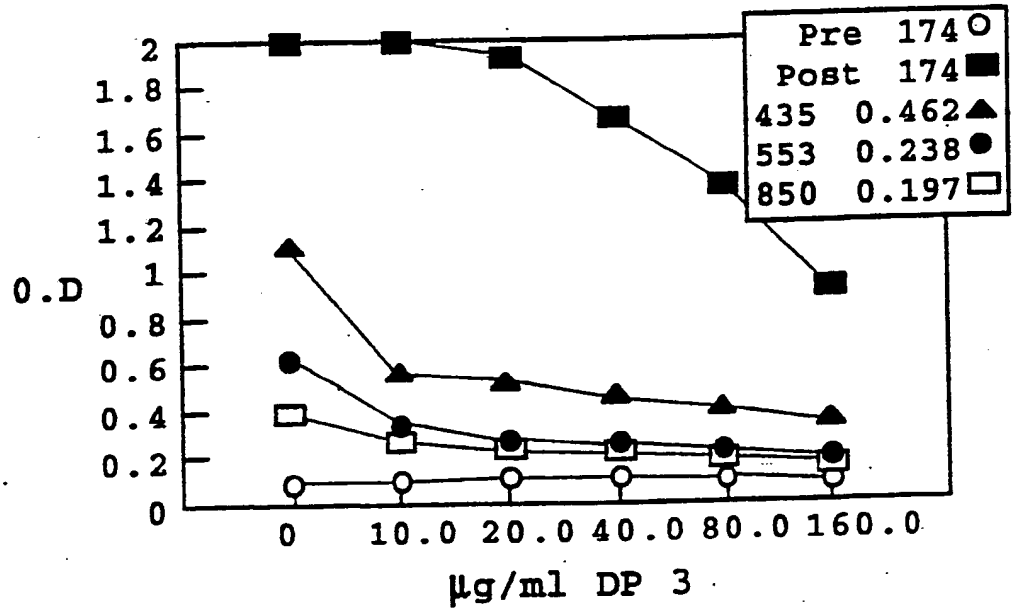



Fig.25B

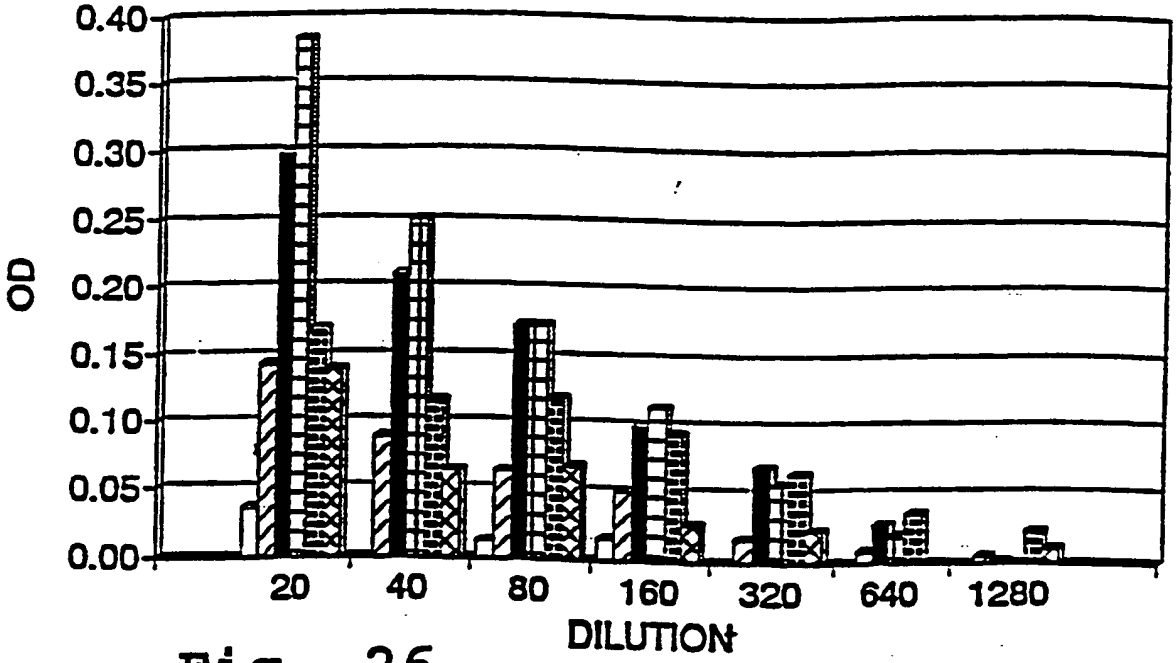
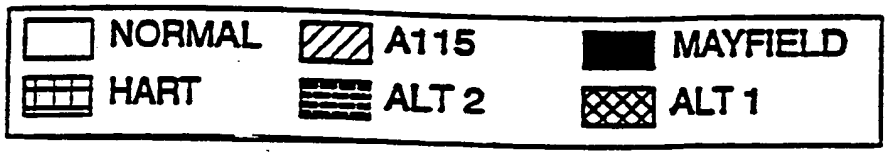

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD
20
40
80
160
320
640
1280
DILUTION

Fig. 26


NORMAL
A115
MAYFIELD
HART
ALT 2
ALT 1

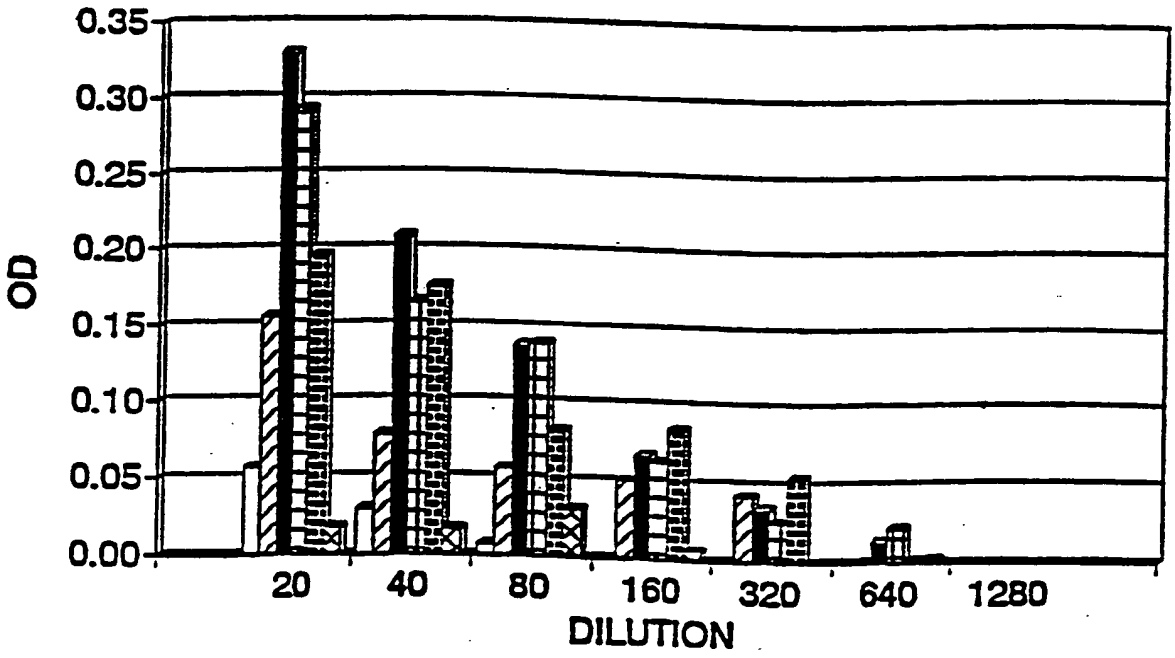

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD
20
40
80
160
320
640
1280
DILUTION

Fig. 27

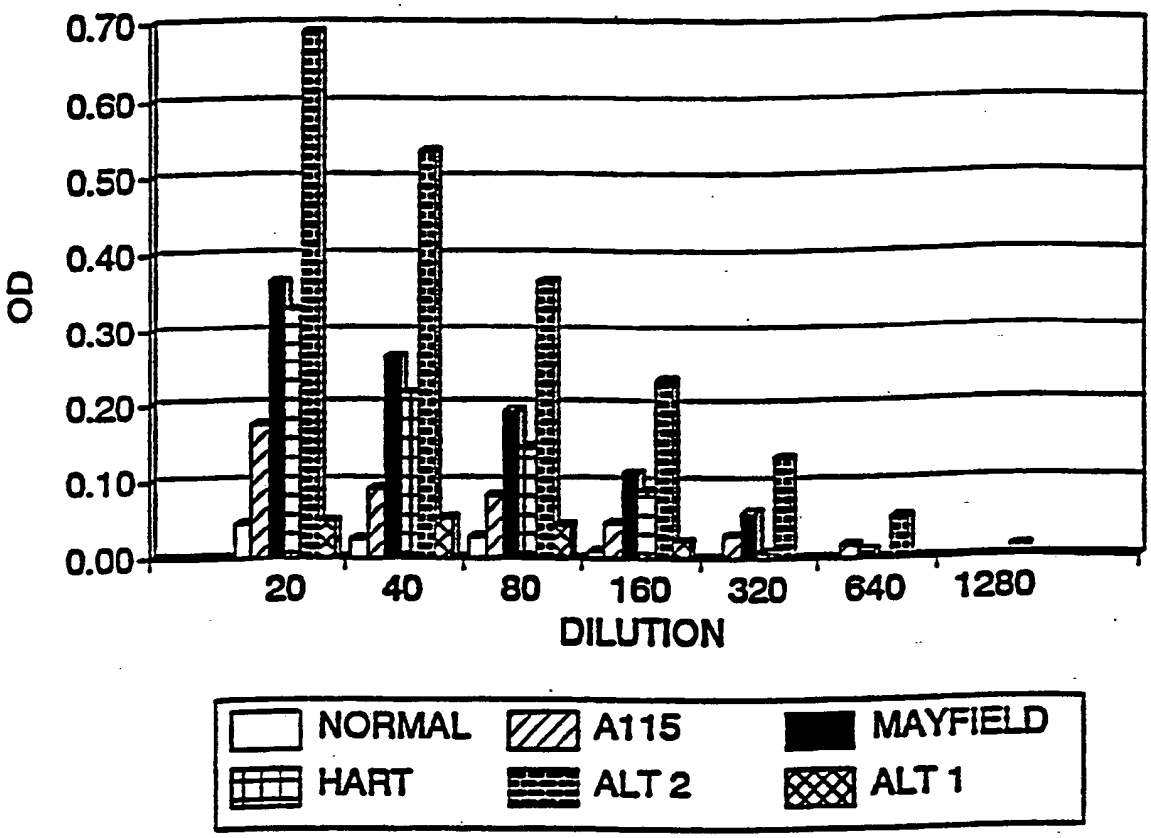

0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OD
20
40
80
160
320
640
1280
DILUTION
NORMAL
A115
MAYFIELD
HART
ALT 2
ALT 1

Fig. 28

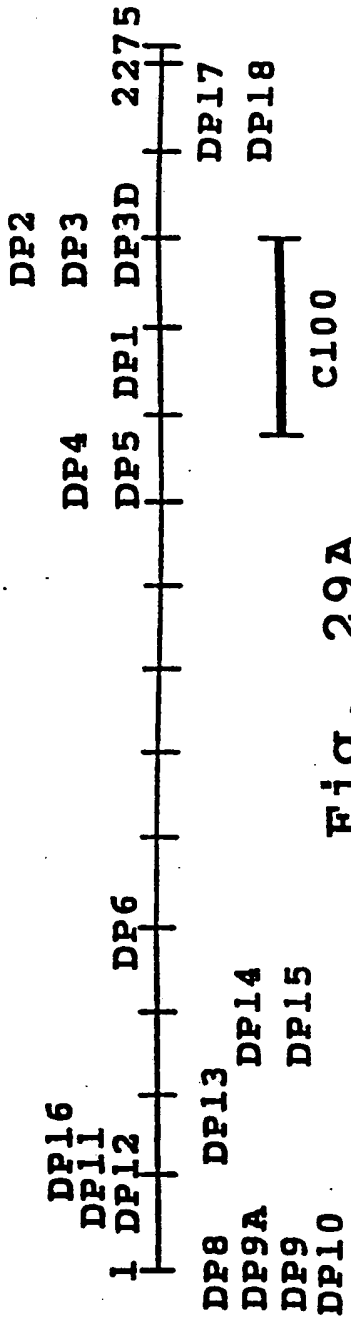



Fig. 29A

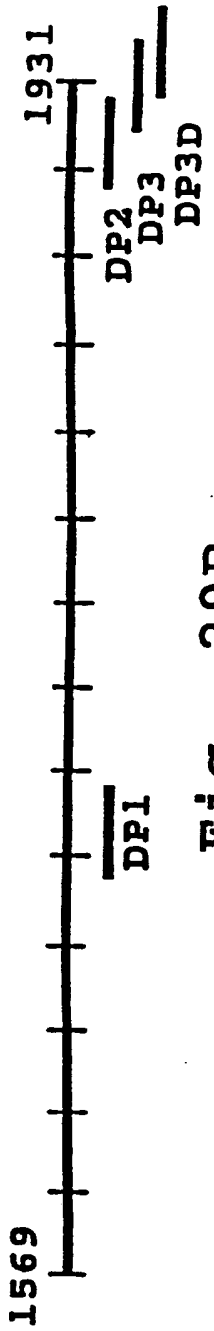



Fig. 29B

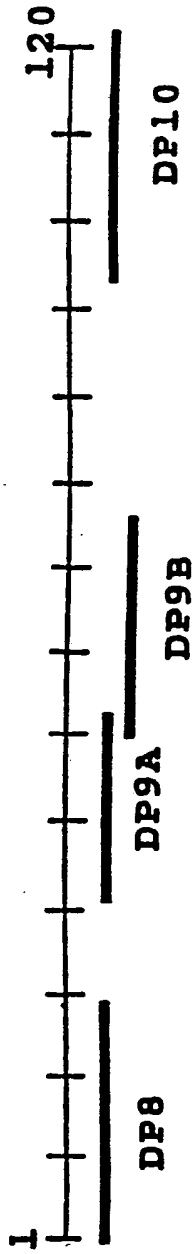



Fig. 29C

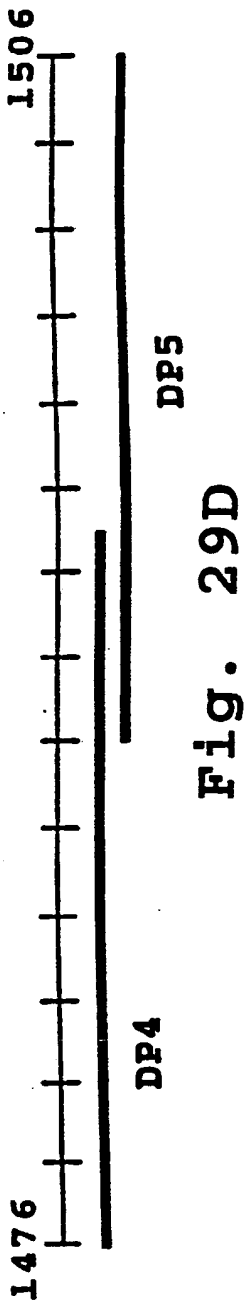



Fig. 29D

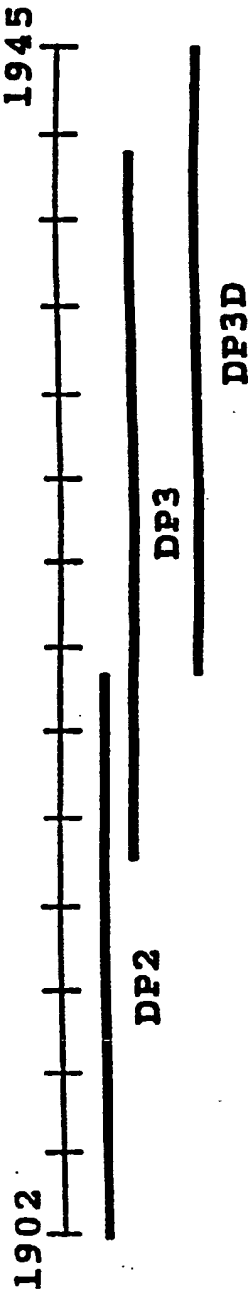



Fig. 29E

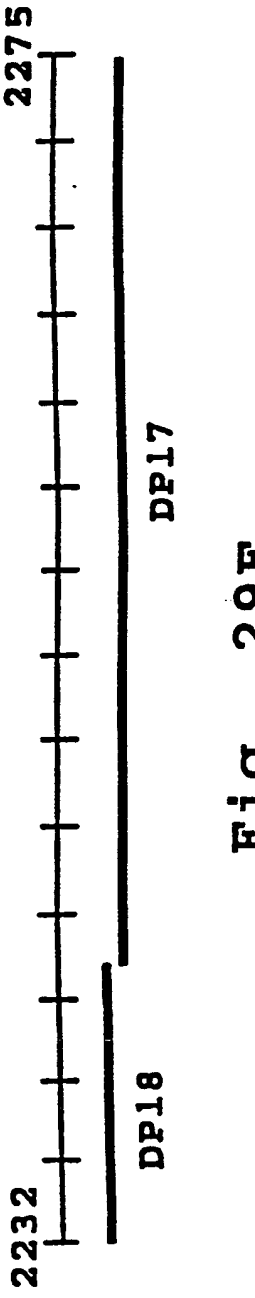



Fig. 29F

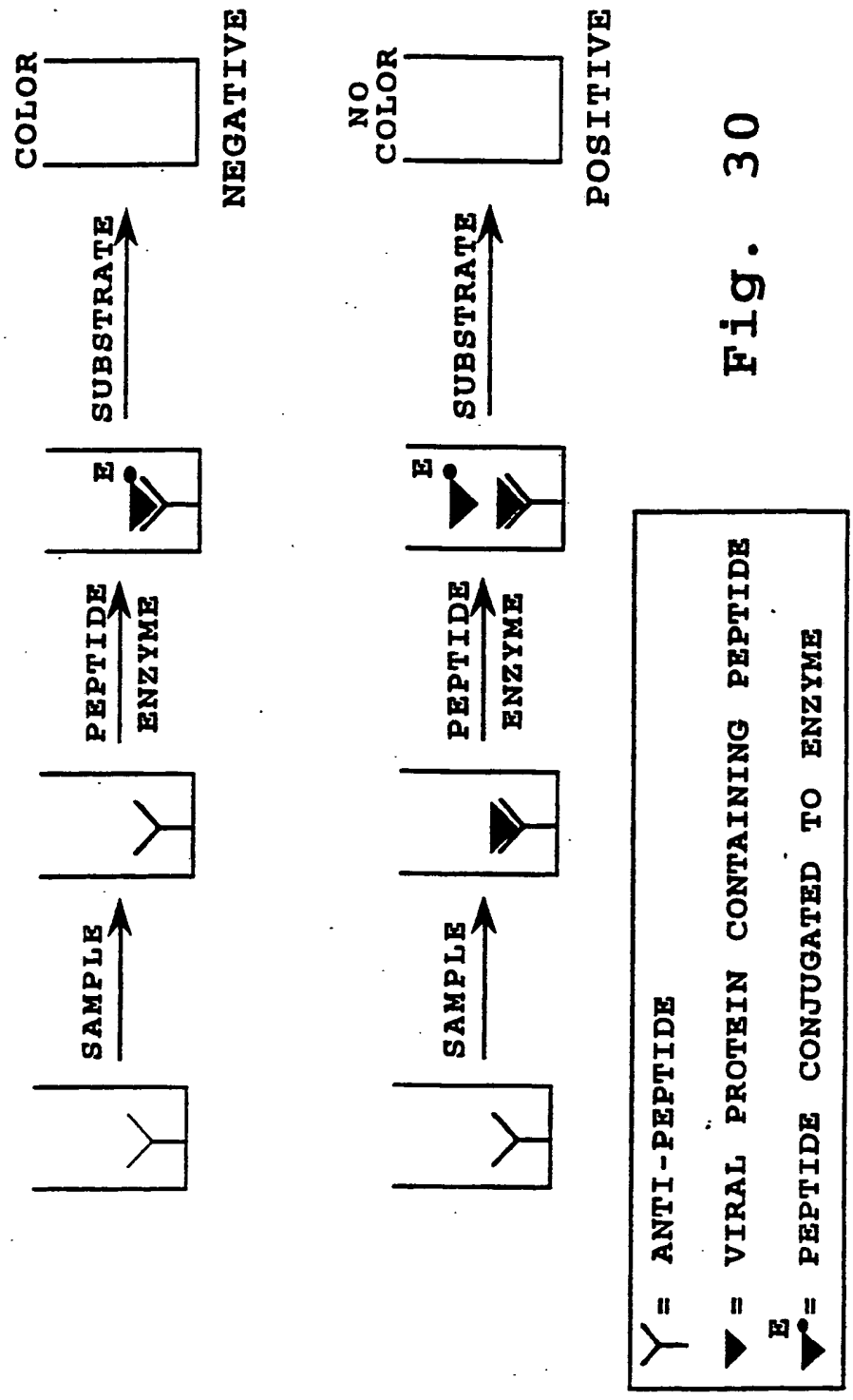

SAMPLE
PEPTIDE ENZYME
SUBSTRATE
COLOR
NEGATIVE
E
SAMPLE
PEPTIDE ENZYME
SUBSTRATE
NO COLOR
POSITIVE
E
= ANTI-PEPTIDE
= VIRAL PROTEIN CONTAINING PEPTIDE
E = PEPTIDE CONJUGATED TO ENZYME

Fig. 30

Fig. 31

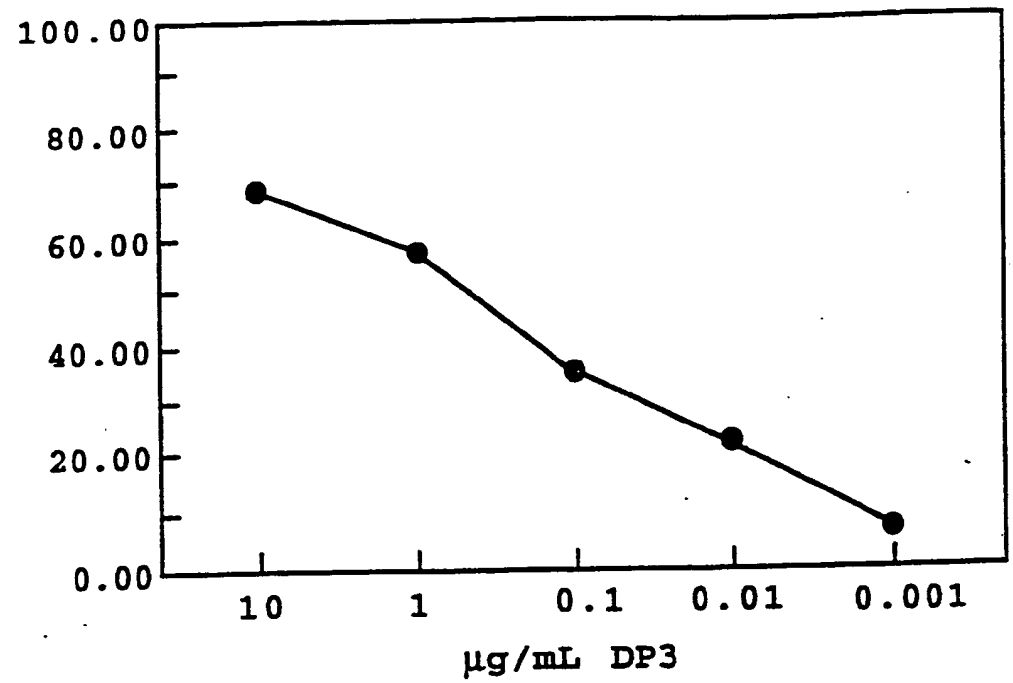
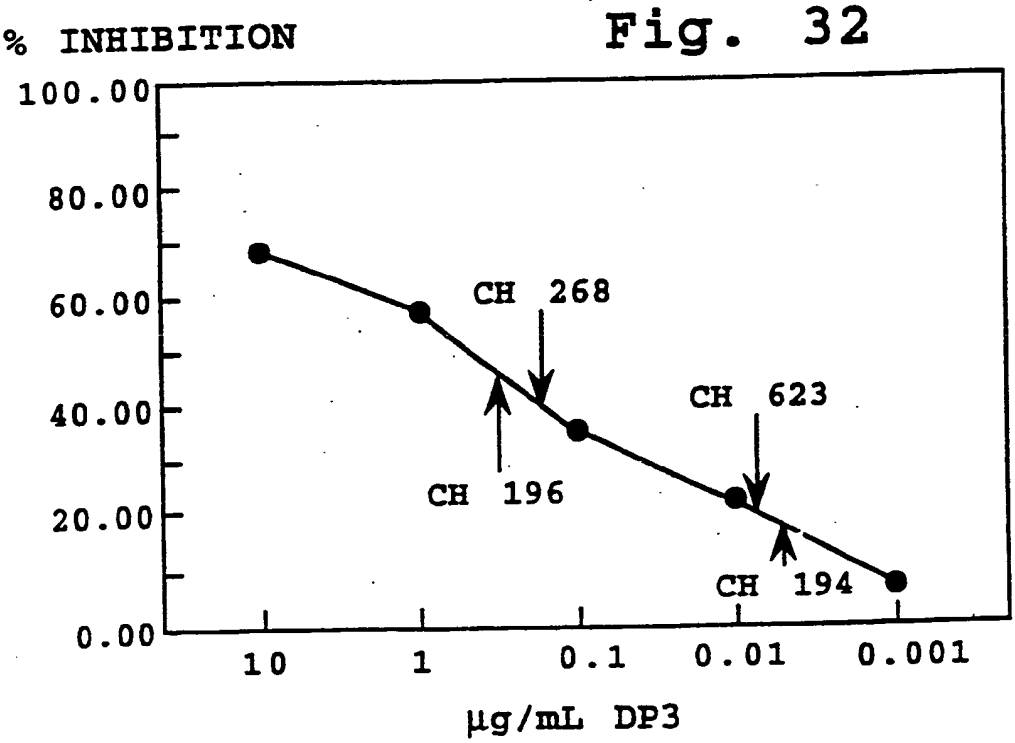

% INHIBITION
100.00
80.00
60.00
40.00
20.00
0.00
10
1
0.1
0.01
0.001
µg/mL DP3

Fig. 32


% INHIBITION
100.00
80.00
60.00
40.00
20.00
0.00
CH 268
CH 623
CH 196
CH 194
10
1
0.1
0.01
0.001
µg/mL DP3

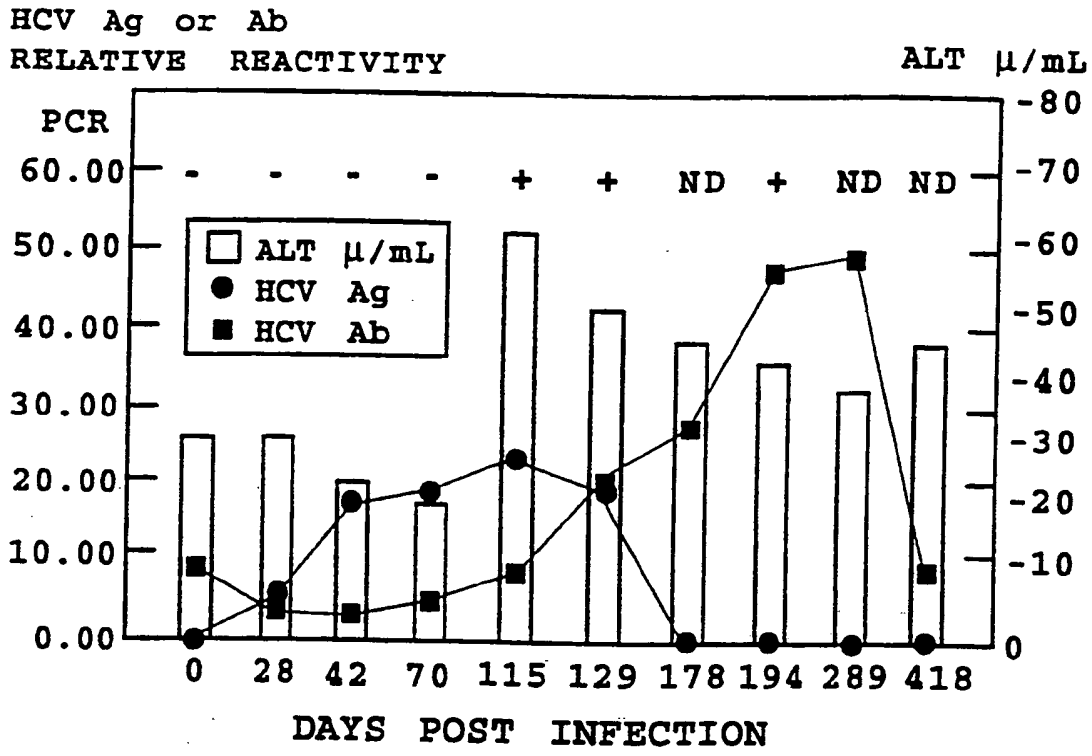

HCV Ag or Ab
RELATIVE REACTIVITY
ALT µ/mL
PCR
60.00
50.00
40.00
30.00
20.00
10.00
0.00
- - - - + + ND + ND ND
ALT µ/mL
HCV Ag
HCV Ab
-80
-70
-60
-50
-40
-30
-20
-10
0
0 28 42 70 115 129 178 194 289 418
DAYS POST INFECTION

Fig. 33

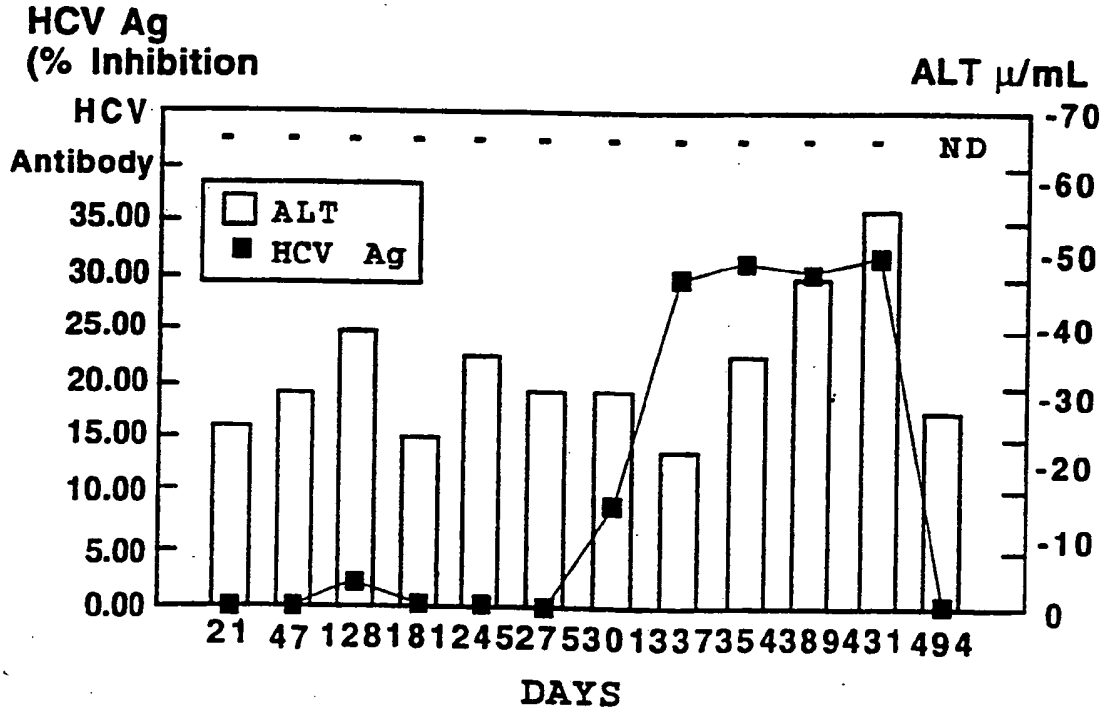

HCV Ag
(% Inhibition
ALT µ/mL
HCV
Antibody
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
- - - - - - - - - - ND
ALT
HCV Ag
-70
-60
-50
-40
-30
-20
-10
0
21 47 128 181 245 275 301 337 354 389 431 494
DAYS

Fig. 34

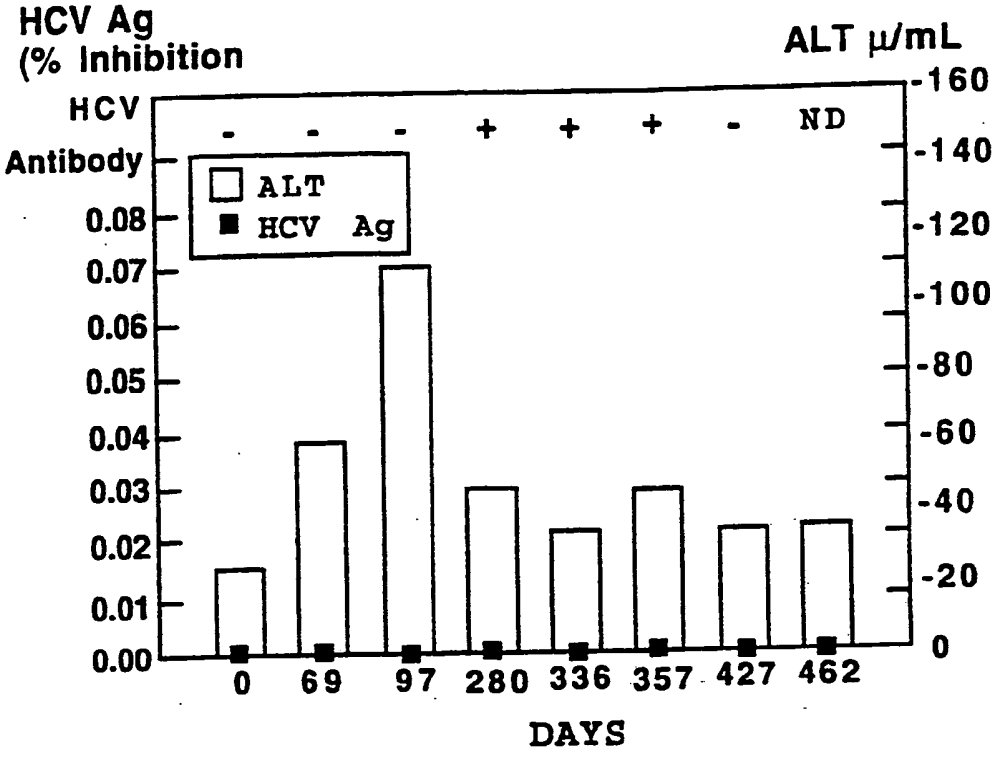



Fig. 35A

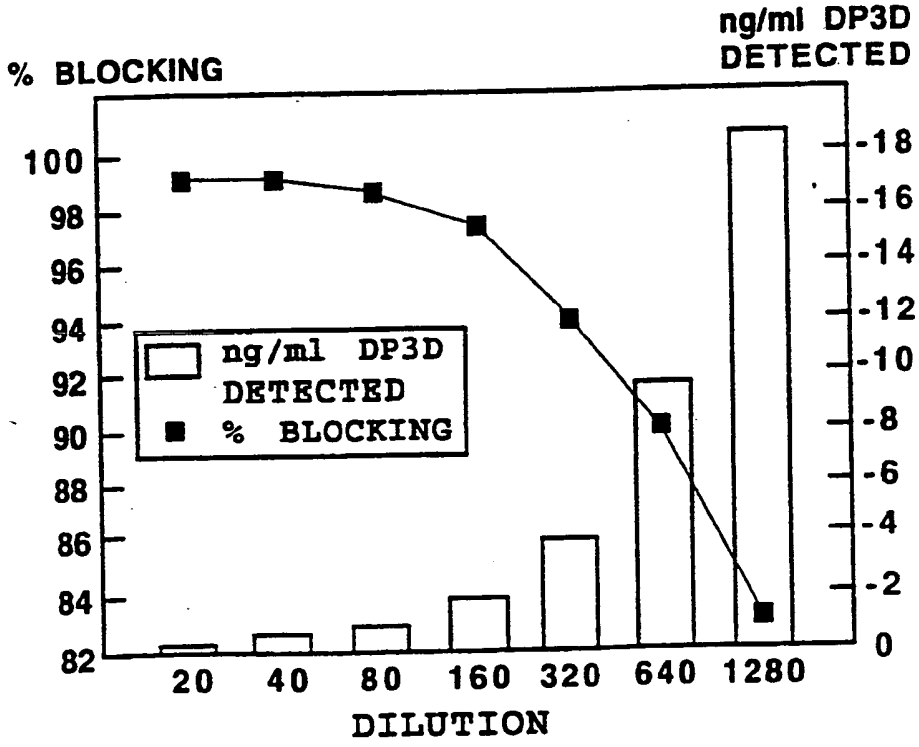



Fig. 35B

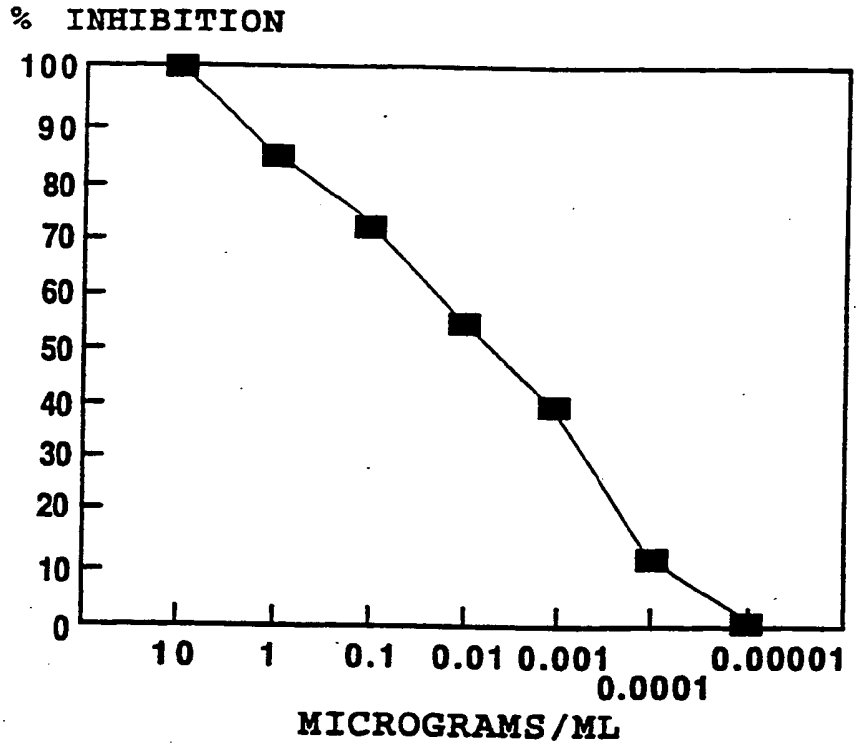



Fig. 35C

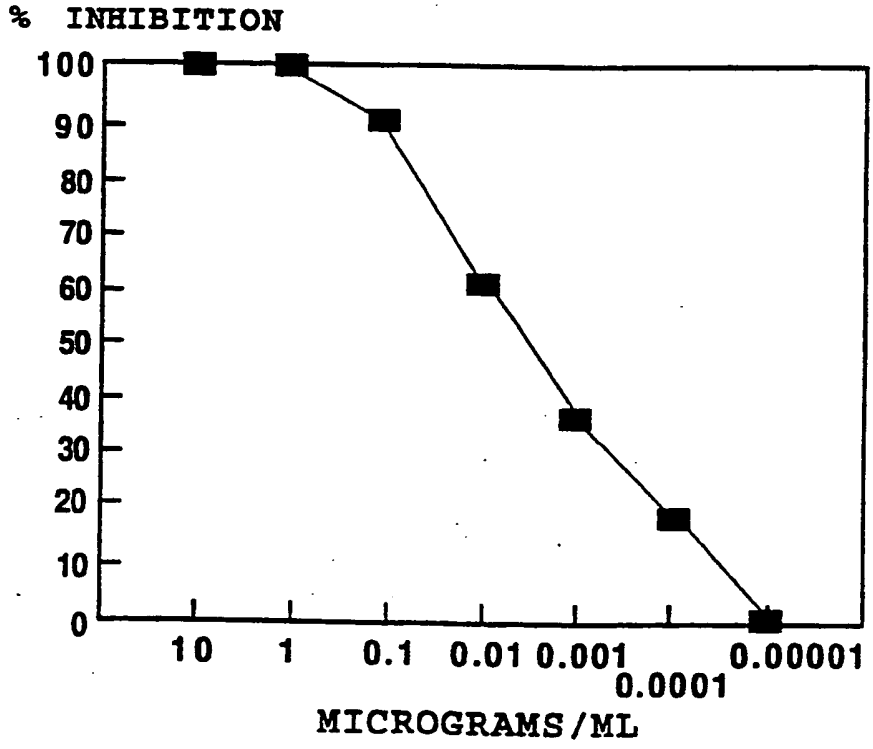



Fig. 41

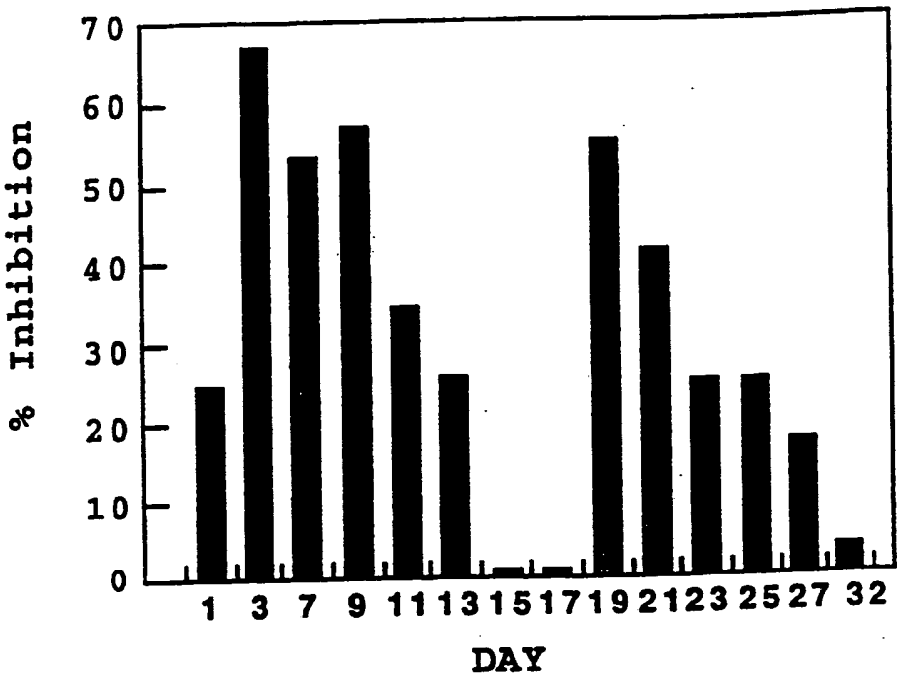

% Inhibition
70
60
50
40
30
20
10
0
1 3 7 9 11 13 15 17 19 21 23 25 27 32
DAY

Fig. 36

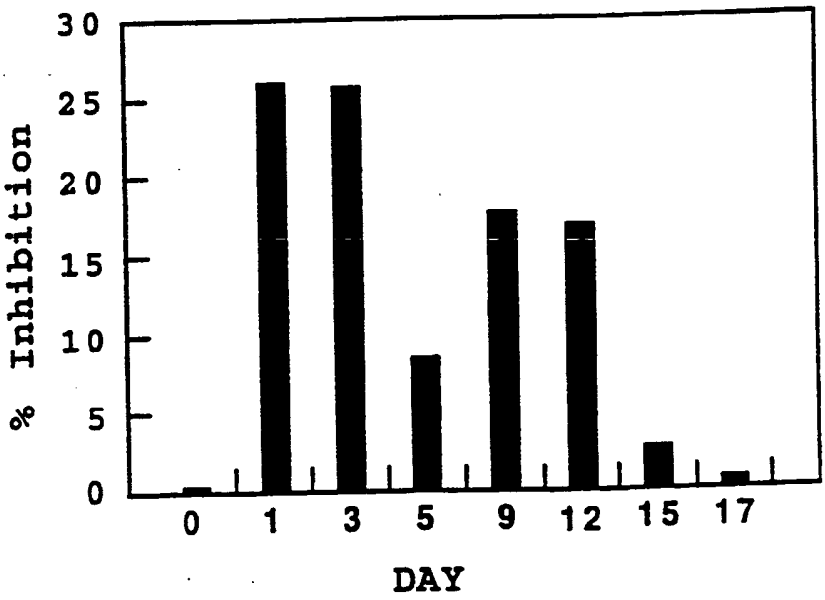

% Inhibition
30
25
20
15
10
5
0
0 1 3 5 9 12 15 17
DAY

Fig. 37

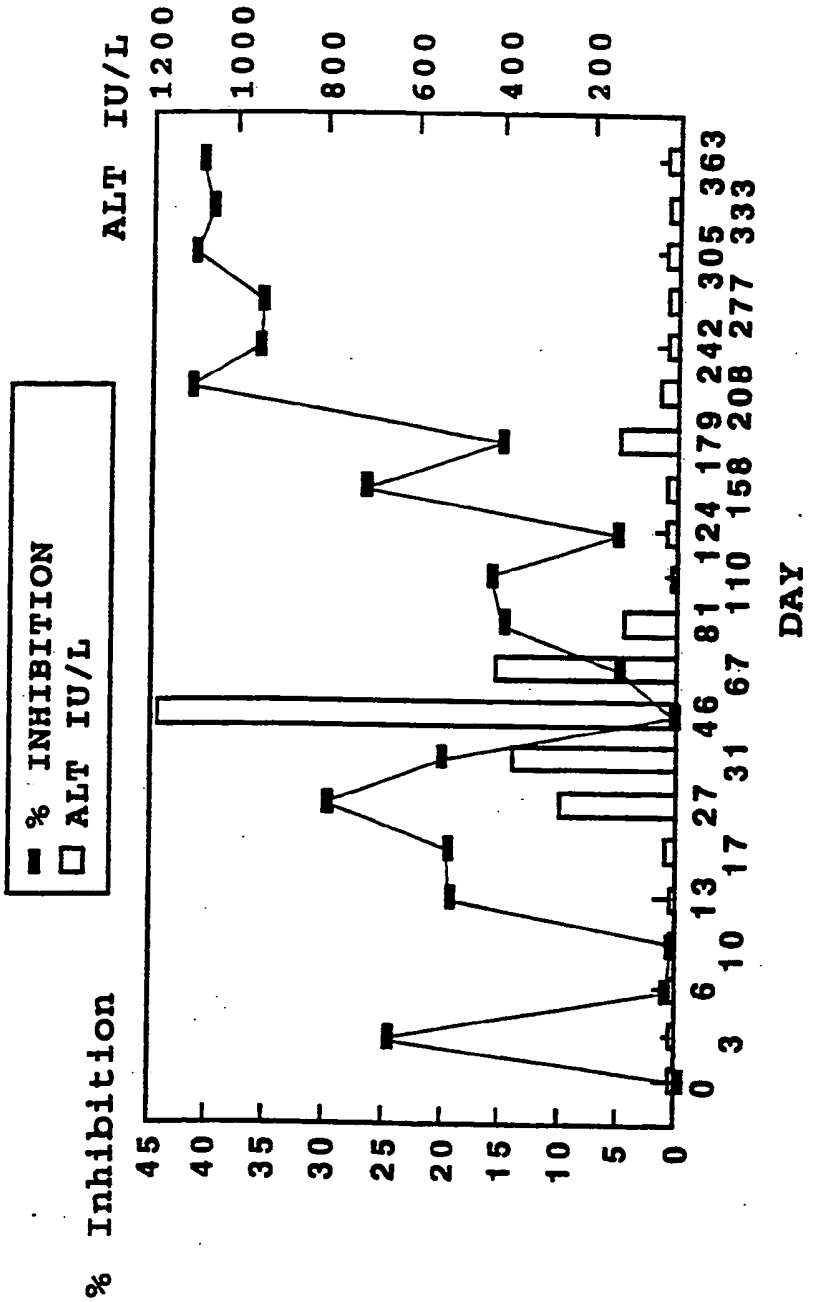

% INHIBITION
ALT IU/L
% Inhibition
ALT IU/L
DAY
0
5
10
15
20
25
30
35
40
45
200
400
600
800
1000
1200
0
3
6
10
13
17
27
31
46
67
81
110
124
158
179
208
242
277
305
333
363

Fig. 38A

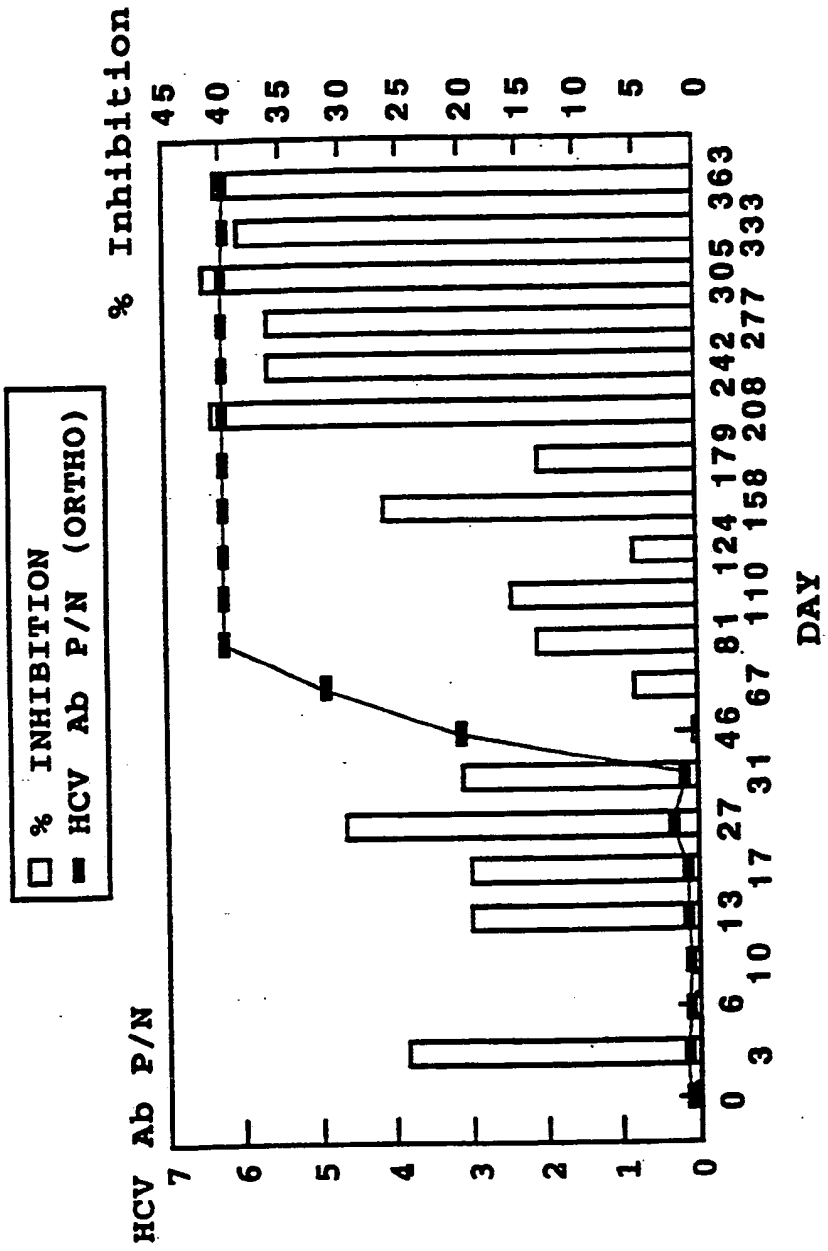

% INHIBITION
HCV Ab P/N (ORTHO)
% Inhibition
HCV Ab P/N
DAY
0
3
6
10
13
17
27
31
46
67
81
110
124
158
179
208
242
277
305
333
363
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
45

Fig. 38B

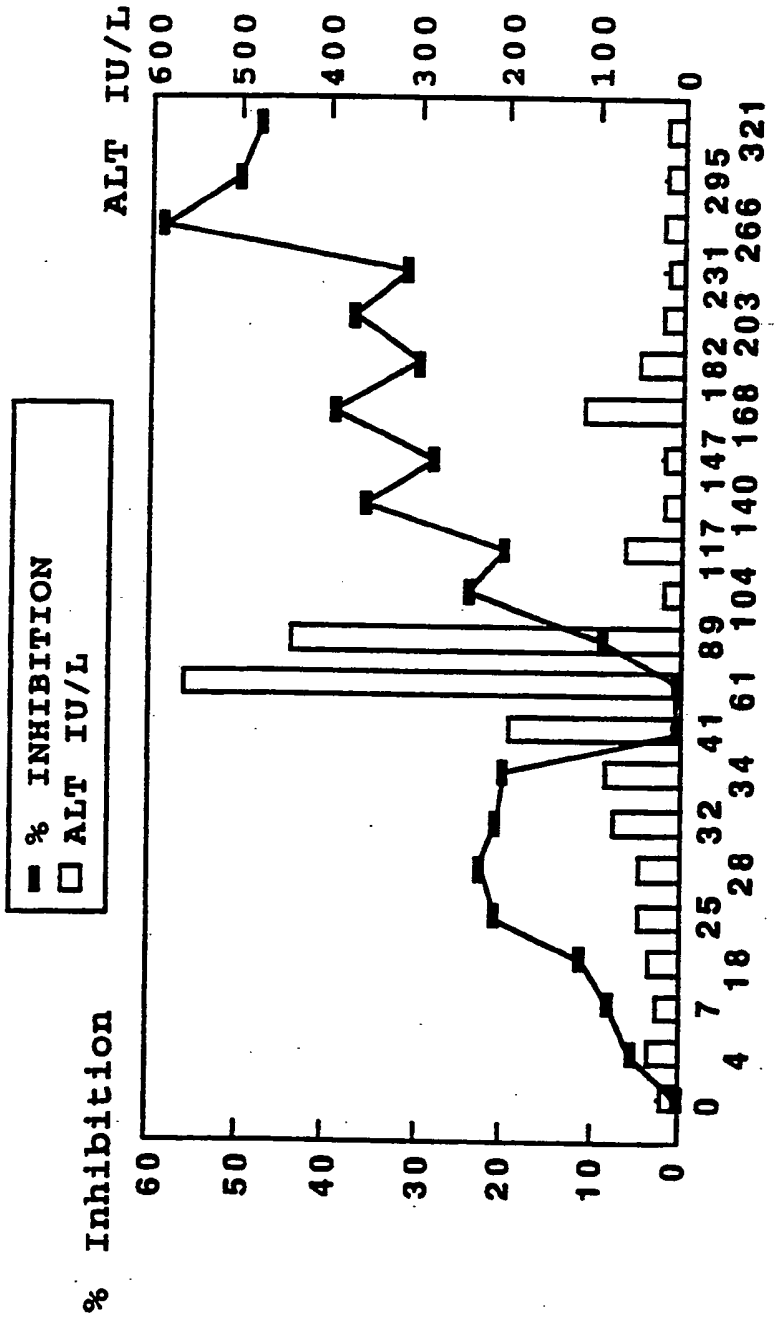

% INHIBITION
ALT IU/L
% Inhibition
ALT IU/L
DAY
0
10
20
30
40
50
60
100
200
300
400
500
600
4
7
18
25
28
32
34
41
61
89
104
117
140
147
168
182
203
231
266
295
321

Fig. 39A

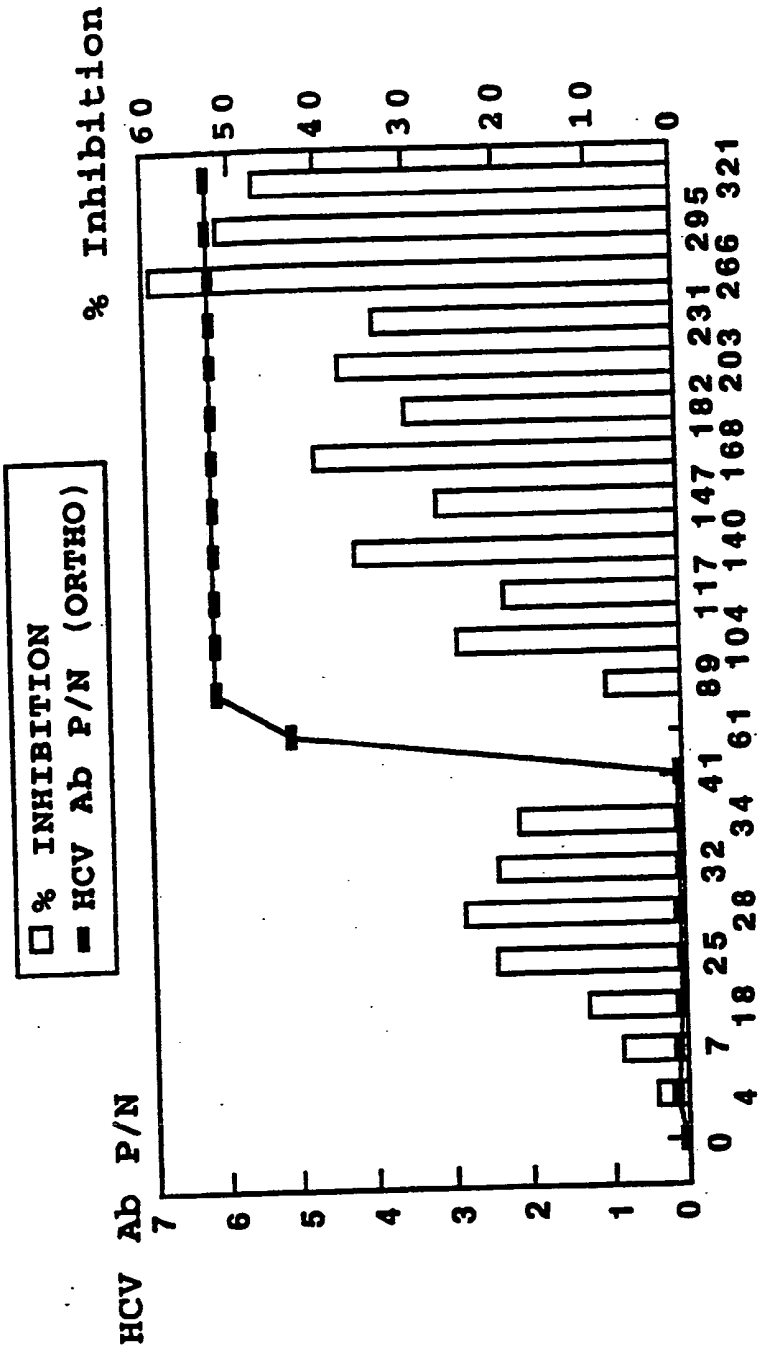

% Inhibition
60
50
40
30
20
10
0
HCV Ab P/N
7
6
5
4
3
2
1
0
□ % INHIBITION
▬ HCV Ab P/N (ORTHO)
0 4 7 18 25 28 32 34 41 61 89 104 117 140 147 168 182 203 231 266 295 321
DAY

Fig. 39B

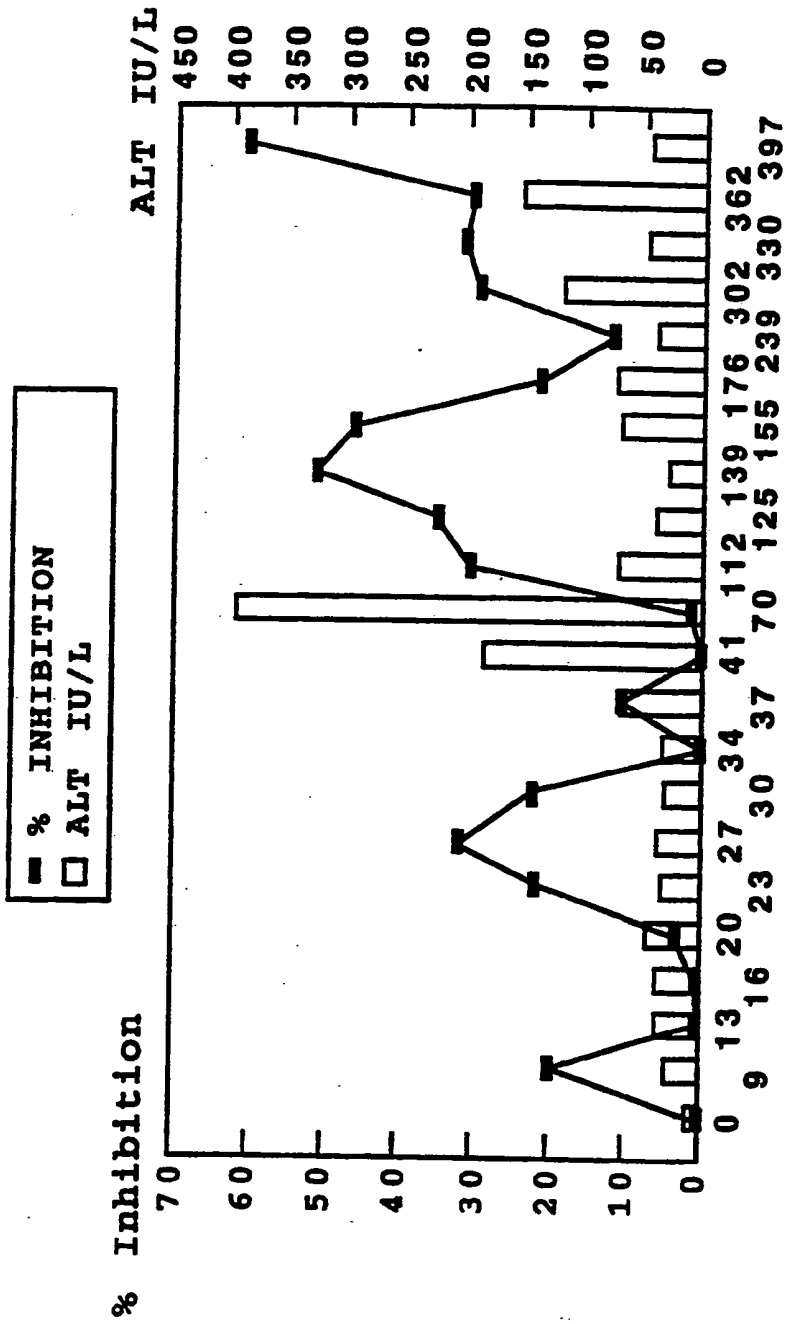

% INHIBITION
ALT IU/L
% Inhibition
ALT IU/L
DAY
0
10
20
30
40
50
60
70
0
50
100
150
200
250
300
350
400
450
0
9
13
16
20
23
27
30
34
37
41
70
112
125
139
155
176
239
302
330
362
397

Fig. 40A

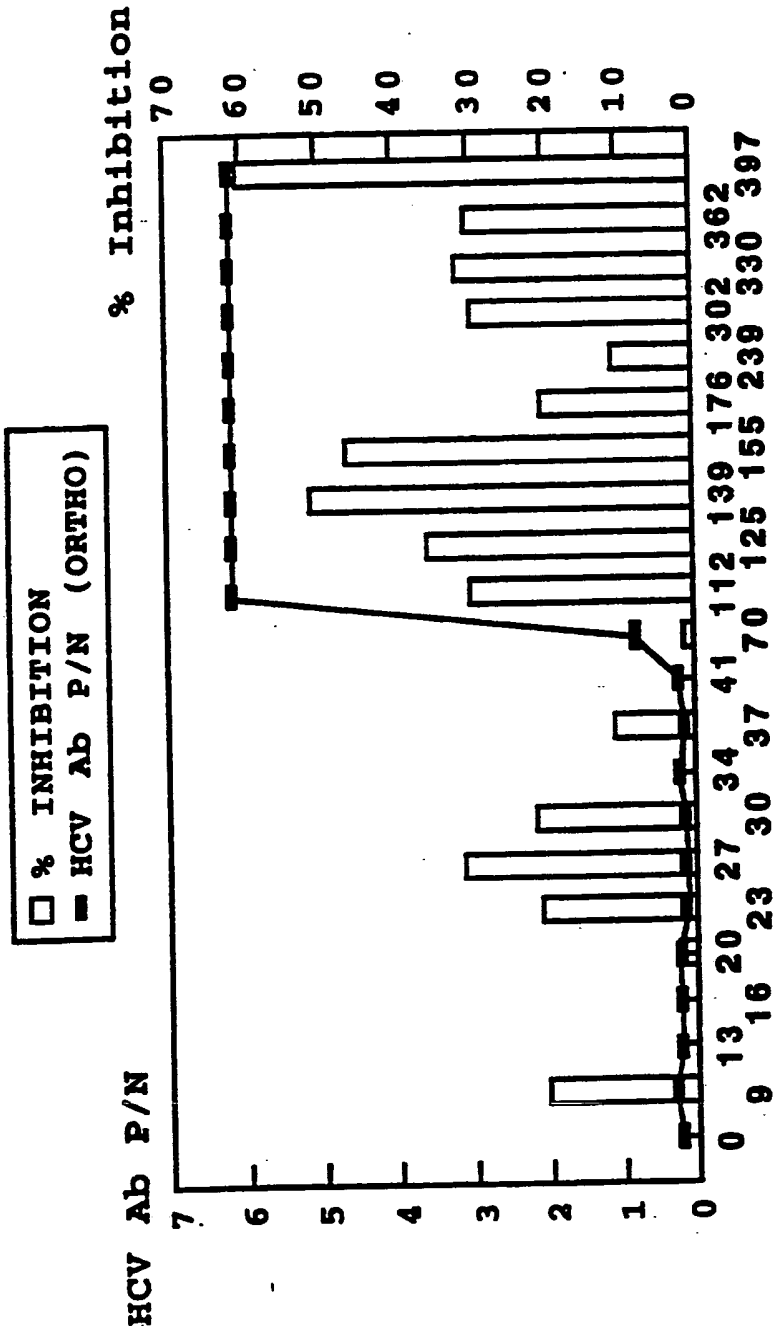

% Inhibition
70
60
50
40
30
20
10
0
HCV Ab P/N
7
6
5
4
3
2
1
0
□ % INHIBITION
■ HCV Ab P/N (ORTHO)
0 9 13 16 20 23 27 30 34 37 41 70 112 125 139 155 176 239 302 330 362 397
DAY

Fig. 40B

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/07865

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/576; C07K15/00

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | G01N ; C07K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| X | EP,A,0 388 232 (CHIRON CORPORATION) 19 September 1990 cited in the application see table on page 34; see page 21, line 21 - line 27; figures 1-17 | 1-4 |
| A | EP,A,0 442 394 (UNITED BIOCHEMICAL INC) 21 August 1991 see the whole document | 1-36 |

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 13 JANUARY 1993 | 28.01.93 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | VAN BOHEMEN C.G. |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9207865
SA 65193

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 13/01/93

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